

REMARKS

Claims 12-17 are pending in the application. Claim 13-17 are withdrawn from consideration. Claim 12 is rejected.

Claim 12 has been amended to delete options (a) to (c). Claim 12 has also been amended per the Examiner's suggestion to recite that the antibody is isolated.

A. Objections to the Specification

The Examiner required that the cross reference to related applications be updated to indicate that the parent application is now U.S. Patent 6,716,613. The Examiner asserted that the title of the invention is not descriptive and required a new title. The Examiner pointed out that the specification does not have the preferred arrangement and required correction.

Submitted herewith is a marked up copy and a clean copy of a substitute specification addressing the issues raised by the Examiner.

No new matter is added to the substitute specification.

B. Claim Rejections - 35 U.S.C. § 112, second paragraph

Claim 12 was rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

The Examiner asserted that Claim 12 is indefinite in reciting sequences "represented by" certain amino acids of SEQ ID NO: 1.

This rejection is moot in view of the amendments to Claim 12.

C. Claim Rejections - 35 U.S.C. § 112, first paragraph

Claim 12 was rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement.

The Examiner asserted that certain phrases in the claims make the claims so broad, that one of ordinary skill in the art would not know how to make functional antibody. More specifically, the Examiner asserted that the phrase “represented by” is open ended, and, thus the claim encompasses antibodies made to polypeptides other than the metalloprotease having SEQ ID NO:1. The Examiner also asserted that the language allowing “1 to 10 amino acid residues” to be “substituted, deleted, and/or inserted” and the language including sequences having “90% or more sequence homology” to SEQ ID NO:1, is so broad that one of ordinary skill in the art could not have contemplated all of the possible sequences encompassed by the claims. Thus, the claims include antibodies to unknown antigens.

The Examiner also asserted that the specification does not teach how to make the claimed antibody, because the antibody must be able to react with a metalloprotease having SEQ ID NO:1, as well as metalloproteases having sequences other than SEQ ID NO:1.

This rejection is moot in view of the amendments to Claim 12.

D. Claim Rejections - 35 U.S.C. § 101

Claim 12 was rejected under 35 U.S.C. § 101 because the claimed invention is directed to non-statutory subject matter.

The Examiner noted that the claim should recite that the antibody is "purified."

Claim 12 has been amended accordingly.

E. Claim Rejections - 35 U.S.C. § 103(a)

Claim 12 was rejected under 35 U.S.C. § 103(a) as being unpatentable over Kuno et al. (JBC 1997. 272; 1: 556-562) in view of Campbell (Monoclonal Antibody Technology. 1984. Published by Elsevier Science Publishers. Pager 1-32), Bost et al. (Immunol. Invest. 1988; 17: 577-586) and Bendayan (J. Histochem. Cytochem. 1995; 43: 881-886)

The Examiner asserted that Kuno et al. disclose a metalloproteinase that has aggrecanase activity. Further, according to the Examiner, the amino acid region from 249-633 of the metalloproteinase has 63% sequence identity with amino acids 214-583 of SEQ ID NO:1. The Examiner admitted that Kuno et al. does not disclose any antibody to the metalloproteinase.

Campbell was cited as showing that it was routine at the time of the invention to make antibodies to isolated proteins. Bost et al. and Bendayan were cited as teaching that an antibody that is specific to a particular sequence of a protein can specifically react with other proteins containing that sequence.

The Examiner concluded that one of ordinary skill in the art, wanting to make a monoclonal antibody to the metalloproteinase of claim 12 would have readily been able to do so using the metalloproteinase of Kuno et al.

For the following reasons, the rejection is traversed and/or overcome.

The attached figure shows a comparison of the sequences of the present invention, the cited reference, and the ortholog of the reference sequence.

hADAMTS1: 253-724
mADAMTS1: 254-725
rADAMTS1: 253-724
MDTS6 (the present invention): 213-682

The relatively long amino sequence region at which the sequence of the reference and the sequence of the present invention match each other corresponds to the region at which the sequence is conserved in ADAMTS1's in different species.

As shown in the Declerck, P. et al. (J.B.C., Vol. 270, No. 15, pp. 8397-8400, 1995- copy submitted herewith), antibodies cannot be generally produced against the auto-antigen and against the highly conserved domains. This is also supported by the following fact. An antiserum which was prepared by immunizing a rabbit with the human u-PA did not show cross immunity to the mouse u-PA having 81% homologous sequence (cf. Table III).

Accordingly, by the conventional method, it is not possible to prepare an antibody against the highly conserved region of ADAMTS1 of different species. This means that the conventional method cannot provide an antibody against the relatively long amino acid sequence region at which the sequence of the reference and the sequence of the present invention match each other.

Accordingly, the antibody of the present invention is not taught or suggested by the cited reference.

In view of the above remarks, the Examiner is requested, respectfully, to reconsider and withdraw the rejection.

AMENDMENT UNDER 37 C.F.R. § 1.111
U.S. Appln. No.: 10/763,210

Atty. Docket No.: Q79353

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

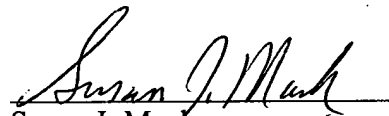
Respectfully submitted,

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON OFFICE

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CUSTOMER NUMBER


Susan J. Mack
Registration No. 30,951

Date: November 22, 2006

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FVSSPRYVETMLVADQSMADFHGSGGLKHYLLTLFVSAAARLYKHPsirnsislVVVKILVIYDEQKGPEVT Majority

1 FVSI PRYVETLV VADQSMADFHGSGGLKHYLLTLFVSAAARLYKHPsirnsislVVVKILVIYDEQKGPEVT Majority
1 FVSS HRYVETMLVADQSMADFHGSGGLKHYLLTLFVSAAARLYKHPsirnsislVVVKILVIYDEQKGPEVT Majority
1 FVSS PRYVETMLVADQSMADFHGSGGLKHYLLTLFVSAAARLYKHPsirnsislVVVKILVIYDEQKGPEVT Majority
1 FVSS PRYVETMLVADQSMADFHGSGGLKHYLLTLFVSAAARLYKHPsirnsislVVVKILVIYDEQKGPEVT Majority

SNAAALTLRNFCNWQKQHNSPSDRDPPEHYDTAILFTRQDLCGSHTCDTLGMADVGTCDPSPRSCSVIEDDG Majority

71 GNAALTLRNFCNWQKQHNSPSDRDPPEHYDTAILFTRQDLCGSHTCDTLGMADVGTCDPSPRSCSVIEDDG Majority
71 SNAAALTLRNFCNWQKQHNSPSDRDPPEHYDTAILFTRQDLCGSHTCDTLGMADVGTCDPSPRSCSVIEDDG Majority
71 SNAAALTLRNFCNWQKQHNSPSDRDPPEHYDTAILFTRQDLCGSHTCDTLGMADVGTCDPSPRSCSVIEDDG Majority
71 SNAAALTLRNFCNWQKQHNSPSDRDPPEHYDTAILFTRQDLCGSHTCDTLGMADVGTCDPSPRSCSVIEDDG Majority

LQAAFTTAHELGHVFNMPHDDAKKHACASLNGVXGDSHLMASMLSSLDHSPWSPCSAYMVTSLFDNGHGEC Majority

141 LPSAFTTAHELGHVFNMPHDDAKKHACASLNGVXGDSHLMASMLSSLDHSPWSPCSAYMVTSLFDNGHGEC Majority
141 LQAAFTTAHELGHVFNMPHDDAKKHACASLNGVXGDSHLMASMLSSLDHSPWSPCSAYMVTSLFDNGHGEC Majority
141 LQAAFTTAHELGHVFNMPHDDAKKHACASLNGVXGDSHLMASMLSSLDHSPWSPCSAYMVTSLFDNGHGEC Majority
141 LQAAFTTAHELGHVFNMPHDDAKKHACASLNGVXGDSHLMASMLSSLDHSPWSPCSAYMVTSLFDNGHGEC Majority

LMDKPQNPIKLPISDLPGTLYDANRQCQFTFGEESKHCPDAASTCTLWCTGTSGGLLVLCQTKHFPWADGT Majority

211 LLDQPSKPIISLPEIDLPGASYTLSQCELAFLGVGSKPCP-YMQYCTKLWCTGTSGGLLVLCQTKHFPWADGT Majority
211 LMDKPQNPIKLPISDLPGTLYDANRQCQFTFGEESKHCPDAASTCTLWCTGTSGGLLVLCQTKHFPWADGT Majority
211 LMDKPQNPIKLPISDLPGTLYDANRQCQFTFGEESKHCPDAASTCTLWCTGTSGGLLVLCQTKHFPWADGT Majority
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SCGEGKWCVSGKCVNKTDMKHFAFATPVHGSWGPWGPWGDCSRTCGGGVQYTMRECDNPVPKNGGKYCEGKR Majority

280 SCGEGKWCVSGKCVNKTDMKHFAFATPVHGSWGPWGPWGDCSRTCGGGVQYTMRECDNPVPKNGGKYCEGKR Majority
281 SCGEGKWCVSGKCVNKTDMKHFAFATPVHGSWGPWGPWGDCSRTCGGGVQYTMRECDNPVPKNGGKYCEGKR Majority
281 SCGEGKWCVSGKCVNKTDMKHFAFATPVHGSWGPWGPWGDCSRTCGGGVQYTMRECDNPVPKNGGKYCEGKR Majority
281 SCGEGKWCVSGKCVNKTDMKHFAFATPVHGSWGPWGPWGDCSRTCGGGVQYTMRECDNPVPKNGGKYCEGKR Majority

VYRSCNLEDCPDNN - GKTFRREEQCEAHNEFSKASFGNEPAVEWTPKYAGVSPKDRCKLICEAKGIGYFF Majority

348 VKYRSCNLEDCPDNN - GKTFRREEQCEAHNEFSKASFGNEPAVEWTPKYAGVSPKDRCKLICEAKGIGYFF Majority
351 VYRSCNLEDCPDNN - GKTFRREEQCEAHNEFSKASFGNEPAVEWTPKYAGVSPKDRCKLICEAKGIGYFF Majority
351 VYRSCNLEDCPDNN - GKTFRREEQCEAHNEFSKASFGNEPAVEWTPKYAGVSPKDRCKLICEAKGIGYFF Majority
351 VYRSCNLEDCPDNN - GKTFRREEQCEAHNEFSKASFGNEPAVEWTPKYAGVSPKDRCKLICEAKGIGYFF Majority

VLQPKVVDGTPCSPDSTSVQVQGCVKAGCDRIIDSKKKFKDKCGVCGNGSTC Majority

418 VLA PKVVDGTPCSPDSTSVQVQGCVKAGCDRIIDSKKKFKDKCGVCGNGSTC Majority
420 VLQ PKVVDGTPCSPDSTSVQVQGCVKAGCDRIIDSKKKFKDKCGVCGNGSTC Majority
420 VLQ PKVVDGTPCSPDSTSVQVQGCVKAGCDRIIDSKKKFKDKCGVCGNGSTC Majority
420 VLQ PKVVDGTPCSPDSTSVQVQGCVKAGCDRIIDSKKKFKDKCGVCGNGSTC Majority

Generation of Monoclonal Antibodies against Autologous Proteins in Gene-inactivated Mice*

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Paul J. Declercq†, Peter Carmeliet,
Maria Verstreken, Frans De Cock,
and Désiré Collen§

From the Center for Molecular and Vascular Biology,
University of Leuven, B-3000 Leuven, Belgium

Induction of an immune response is strongly dependent on the phylogenetic distance between antigen and recipient. In general, antibodies will not be raised against self-antigens nor against highly conserved domains. In the present study we describe the production and characterization of murine monoclonal "auto-antibodies" against murine tissue-type plasminogen activator (t-PA) raised in "knock-out" mice, homozygously deficient of the functional gene. 203 stable hybridomas were obtained producing murine monoclonal antibodies against murine t-PA. Analysis of the species reactivity revealed that 182 cross-reacted with one or more (t-)PAs originating from other species including rat t-PA, human t-PA, and vampire bat-PA. 121 reacted with epitopes conserved among murine, rat, and human t-PA. In addition, 31 of the monoclonal antibodies were directed against domains present in all four species. Epitope mapping indicated a high frequency of specificity toward diverse epitopes that are highly conserved across species. Comparative analysis of their influence on the enzymatic activity of t-PA and their species cross-reactivity clearly demonstrated that the domains required for the biological activity of plasminogen activators are more conserved ($p < 0.02$) than non-functional domains.

The availability of such unique antibodies against a wide variety of conserved epitopes may facilitate studies on the structural homologies between (t-)PAs isolated from various species. The present approach should also apply to various other classes of proteins, allowing the generation of monoclonal antibodies, against conserved epitopes, which could not be raised in wild-type animals because of their "self-antigen" nature.

Since the development of the hybridoma technology by Kohler and Milstein (1), the production of murine monoclonal

antibodies against foreign antigens has become a routine technique. However, it is generally accepted that the greater the phylogenetic distance between antigen and recipient, the more pronounced the immune response, e.g. highly conserved mammalian proteins usually evoke a weak immune response (2). In this same view, generation of murine monoclonal antibodies against murine proteins (i.e. self-antigens) is not to be expected due to an absence, by active elimination or functional inactivation, of lymphocytes bearing receptors for self-antigens (3, 4). For similar reasons, the immunological system will also be unable to recognize an epitope present in a protein obtained from another species, but showing a high degree of homology with an epitope in the corresponding protein of the immunized species. As a consequence all available monoclonal antibodies of murine origin, raised against a particular non-murine protein, will not cross-react (or only to a low extent) with the corresponding murine protein. From a practical viewpoint, this eliminates the possibility of detection or quantitation of murine proteins with highly specific murine monoclonal antibodies. More importantly, none of the currently available murine monoclonal antibodies should react with domains or epitopes that are conserved between various species.

We therefore hypothesized that immunization of transgenic mice, in which the expression of specific proteins is abolished by homologous recombination in embryonic stem cells (5), could result in the generation of murine antibodies against the "knocked-out" protein and against epitopes conserved across species. In the present study we describe the generation and characterization of a wide variety of murine monoclonal antibodies raised against murine tissue-type plasminogen activator (mt-PA)¹ in transgenic mice in which the gene encoding tissue-type plasminogen activator had been inactivated (6).

MATERIALS AND METHODS

Monoclonal Antibodies—Monoclonal antibodies were produced essentially as described by Galfré and Milstein (7). Homozygous t-PA-deficient (t-PA^{-/-}) mice (6) were immunized by subcutaneous injection of 10 μ g murine t-PA in complete Freund's adjuvant, followed 2 weeks later by intraperitoneal injection of 10 μ g of mt-PA in incomplete Freund's adjuvant. Antisera were collected 1 week later and were analyzed in a micro-ELISA using microtiter plates coated with mt-PA (1 μ g/ml) and detection of bound immunoglobulins with horseradish peroxidase-conjugated rabbit anti-mouse IgG. The specific antibody concentration in these antisera (see "Results") was retrospectively calculated by ELISA on microtiter plates coated with the respective antigen using purified monoclonal antibodies for calibration. An identical procedure was used for the evaluation of the immune response in wild-type mice. After an interval of at least 4 weeks, the mice were boosted intraperitoneally with 10 μ g of mt-PA in saline on days 4 and 2 before the cell fusion. Spleen cells were isolated and fused with either P3x63Ag.8-6.5.3 or Sp2/0-Ag14 myeloma cells. After selection in hypoxanthine-aminopterin-thymidine medium, the supernatants were screened for specific antibody production with a one-site non-competitive micro-ELISA using microtiter plates coated with mt-PA and detection of bound immunoglobulins as described above. Positive clones were used for the production of ascites in pristane-primed mice. The IgG fraction of the monoclonal antibodies was purified from ascites by affinity-chromatography on protein A-Sepharose. A similar procedure

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† Senior Research Associate of the National Fund for Scientific Research (Belgium). Current address: Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, University of Leuven, B-3000 Leuven, Belgium.

§ To whom correspondence should be addressed: Center for Molecular and Vascular Biology, University of Leuven, Campus Gasthuisberg, O & N, Herestraat 49, B-3000 Leuven. Tel.: 32-16-345772; Fax: 32-16-345990.

¹ The abbreviations used are: mt-PA, murine t-PA; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; ht-PA, human t-PA; rt-PA, rat t-PA; bat-PA, vampire bat plasminogen activator; mu-PA, murine u-PA; hu-PA, human u-PA; t-PA^{-/-}, t-PA-deficient; u-PA^{-/-}, u-PA-deficient; ELISA, enzyme-linked immunosorbent assay.

was followed for the generation of monoclonal antibodies against murine urokinase-type plasminogen activator (mu-PA) in homozygous u-PA-deficient (u-PA^{-/-}) mice.

Cross-reactivity—Cross-reactivity of the obtained anti-mt-PA monoclonal antibodies with (t-)PA from other species (rat, human, or vampire bat) or with murine urokinase-type plasminogen activator, was evaluated by comparative analysis of the reactivity of hybridoma supernatant or purified antibody in a micro-ELISA system using microtiter plates coated with 1 µg/ml of the respective antigen and detection of bound immunoglobulins as described above. Parallel control reactions were carried out using microtiter plates coated with bovine serum albumin to exclude false positive reactions.

Domain Localization—Domain localization of the epitope recognized by antibodies cross-reacting with human t-PA (ht-PA) was evaluated by comparative analysis of their reactivity in various micro-ELISAs using microtiter plates coated with either one of the domain deletion and/or insertion variants of human t-PA (see below).

Determination of Overlapping Epitopes—Determination of overlapping epitopes was carried out through competition of purified antibodies for binding to mt-PA using the Biacore® (Pharmacia, Uppsala, Sweden). Briefly, mt-PA (10 µg/ml, in 10 mM acetate, pH 5.0, 30 µl) was coupled to the sensor chip, followed by incubation with one antibody and evaluation of its binding. Subsequently, the binding of a second antibody was evaluated. Lack of binding of the second antibody indicates that both antibodies are directed against the same epitope, or that a significant portion of the epitopes overlap. All pairs of antibodies evaluated were analyzed in both sequences. After each cycle the sensor chip was regenerated using 10 mM HCl. Positive and negative control samples were included at the beginning and at the end of each set of experiments.

Influence on Enzymatic Activity of t-PA—mt-PA (4 ng/ml) was pre-incubated (1 h at room temperature) with conditioned medium diluted 1:5 or with a 20-fold molar excess of purified monoclonal antibody. Subsequently residual t-PA activity was measured by a plasminogen-coupled chromogenic substrate assay as described (8).

Proteins—Rat t-PA (9), vampire bat-PA (DSPA1) (10), murine t-PA (6), and the human t-PA variants K₁K₂P (lacking the finger-like and growth factor domain) (11), EK₁K₂P (lacking the finger-like domain) (11), K₂K₂P (lacking the finger-like, growth factor, and kringle 1 domains and containing an extra kringle 2 domain) (12), FEK₂K₂P (lacking the kringle 1 domain and containing an extra kringle 2 domain) (12), and K₂P (consisting of kringle 2 and the protease domain of human t-PA) (13) were produced and characterized as described. A hybrid molecule (K₁K₂P_{1-PA}) consisting of kringle 1 and kringle 2 of human t-PA and the protease part of human u-PA was produced and characterized as described (14). Human t-PA was a kind gift from Genentech Inc. (San Francisco, CA).

Statistical Analysis—The statistical significance of differences was assessed using the Mann-Whitney nonparametric test for unpaired values and Fisher's exact test for proportions. *p* values > 0.05 were considered to be not significant.

RESULTS

Administration of mt-PA evoked a strong immune response in t-PA^{-/-} mice, resulting in a specific antibody concentration in serum of 675 µg/ml (median; range 100–3,000, *n* = 4) against mt-PA and of 100 µg/ml (median; range 25–500, *n* = 4) against human t-PA (ht-PA). Administration of murine urokinase-type plasminogen activator (mu-PA) to urokinase-type plasminogen activator-deficient (u-PA^{-/-}) mice induced a specific antibody concentration of 350 µg/ml serum (median; range 33–2,000, *n* = 4) against mu-PA. However, no significant cross-reactivity was observed with human u-PA (hu-PA). These findings suggest that there are common (conserved) epitopes on mt-PA and ht-PA but not on mu-PA and hu-PA; they are consistent with our previous experience that polyclonal antisera raised in rabbits against ht-PA cross-react with mt-PA, whereas antisera raised against hu-PA do not cross-react with mu-PA.² Administration of mt-PA or mu-PA to wild-type mice resulted in a specific antibody concentration in serum of 15 µg/ml (median; range 9–130, *n* = 4) against mt-PA and 4 µg/ml (median; range 3–30, *n* = 4) against

TABLE I
Cross-reactivity of anti-murine t-PA monoclonal antibodies with t-PA obtained from various species

The data represent the number of monoclonal antibodies that cross-react with t-PA from the respective species. Numbers in parentheses represent the number of monoclonal antibodies that inhibit plasminogen activation by mt-PA.

Reactivity	- Bat-PA	+ Bat-PA
Mouse	21 (6)	1 (0)
Mouse + rat	21 (13)	2 (2)
Mouse + human	6 (3)	0 (0)
Mouse + rat + human	121 (68)	31 (19)

mu-PA, respectively (*p* = 0.029 and *p* = 0.014 versus gene-inactivated mice, respectively).

Two fusions of myeloma cells with spleen cells isolated from immunized t-PA^{-/-} mice yielded 203 hybridomas producing monoclonal antibodies against mt-PA, whereas one fusion with spleen cells of an immunized u-PA^{-/-} mouse yielded 38 hybridomas producing monoclonal antibodies against mu-PA. Of the antibodies against mt-PA, 78% cross-reacted with ht-PA, whereas none of the anti-mu-PA antibodies cross-reacted with hu-PA, confirming the differential cross-species reactivity of polyclonal antisera raised with the respective proteins.

The cross-reactivity of the anti-mt-PA monoclonal antibodies was further studied using rat t-PA (rt-PA) and vampire bat-PA (bat-PA). Table I illustrates that 10% of the monoclonal antibodies reacted exclusively with mt-PA, 10% cross-reacted with rt-PA, 3% with ht-PA, and 0.5% with bat-PA. However, 61% cross-reacted with three of the four PAs tested, while 15% reacted with all four PAs. None of the monoclonal antibodies raised with mt-PA cross-reacted with mu-PA.

All antibodies cross-reacting with ht-PA (*i.e.* *n* = 158) could be further evaluated with respect to their domain reactivity using a set of deletion/insertion mutants of ht-PA. These experiments revealed that the majority of these antibodies were directed against an epitope in kringle 1 (*n* = 48), kringle 2 (*n* = 33), or the protease domain (*n* = 52) (Table II). 16 monoclonal antibodies required the simultaneous presence of at least two domains (K₁K₂, K₁-P, or FEK₁). Only two antibodies were directed against an epitope in the finger-like domain, while none recognized the growth factor-like domain. Further analysis revealed that the epitopes recognized by the subgroup of 31 monoclonal antibodies reacting with all four PAs were exclusively localized in the finger-like, kringle 1, or the protease domain but not in the kringle 2 domain (Table II).

Evaluation of the interference of the monoclonal antibodies with the plasminogen activation potential of mt-PA revealed that 111 out of 203 exhibited inhibitory properties toward mt-PA activity (Table I). Of the 21 monoclonal antibodies reacting exclusively with mt-PA, only 6 (29%) were inhibitory, whereas out of the 182 monoclonal antibodies cross-reacting with one or more other (t-)PAs, 105 (58%) were inhibitory. Table II shows a subanalysis in which the inhibitory properties of the 158 monoclonal antibodies cross-reacting with ht-PA were compared to their domain reactivity in ht-PA. From these data it appeared that the majority (26 out of 33, 81%) of the kringle-2-reacting antibodies had an inhibitory effect while only 35% (17 out of 48) of the kringle-1-reacting antibodies interfered with the activity. 32 out of 52 (62%) monoclonal antibodies reacting with the protease domain exhibited inhibitory properties (Table II). Interestingly, the kringle-1-reacting antibodies that cross-reacted with the four (t-)PAs were all inhibitory.

26 purified monoclonal antibodies were further subjected to a detailed epitope mapping analysis based on their mutual competition for binding to mt-PA. These data revealed the

² P. J. Declerck, M. Verstreken, P. Carmeliet, D. Collen, unpublished observations.

TABLE II
Domain localization of the epitopes recognized by monoclonal antibodies that cross-react with human t-PA

The data represent the number of monoclonal antibodies reactive with the indicated species and with the indicated domains of human t-PA. Numbers in parentheses represent the number of antibodies that inhibit plasminogen activation. Based on the homology between distinct portions of the amino acid sequence of t-PA with corresponding domains of other proteins, five subdomains ("modules") are distinguished: a fingerlike domain (F), an epidermal growth-factor domain (E), two kringle domains (K_1 and K_2), and a protease domain (P). Presentation of one domain indicates that the epitope is localized entirely in the respective domain. Presentation of two or three domains indicates that the epitope is composed of parts of two or three domains. Undefined indicates that the domain reactivity could not be deduced unambiguously. mu, murine; hu, human; ra, rat; ba, vampire bat.

Species reactivity	Domain reactivity							
	F	(FE) K_1	K_1	K_1 - K_2	K_2	P	K_1 P	?
mu/hu	—	—	—	—	3 (0)	1 (1)	—	2 (2)
mu/hu/ra	1 (1)	—	39 (8)	3 (2)	30 (26)	40 (26)	4 (2)	4 (3)
mu/hu/ra/ba	1 (0)	7 (4)	9 (9)	—	—	11 (5)	3 (1)	—

presence of at least six distinct clusters. Fig. 1 shows a schematic diagram of the various epitopes and the association with their domain and/or species reactivity. At least three distinct epitopes were localized in the kringle 1 domain, two in the protease domain and one in the kringle 2 domain. At least three non-overlapping epitopes, two located in the kringle 1 domain and one located in the protease domain, were conserved in all four species and are involved in the t-PA activity.

DISCUSSION

In general, antibodies are prepared either from antisera collected from immunized animals or by hybridoma technology using spleen cells from immunized mice and formation of stable hybridomas producing monoclonal antibodies. Recently strategies have been developed for expression of antibody fragments in *Escherichia coli* and for the generation of combinatorial libraries in bacteriophages, using phagemid vectors allowing the formation of "phage-displayed" antibodies (15, 16). The efficiency (i.e. number of antibodies as well as their affinity) of the latter procedures is also dependent on the immunization of the animal prior to construction of the library (15). In addition, different approaches have been described to mimic, *in vitro*, the naturally occurring process of affinity maturation (17, 18). However, because of the clonal selection, resulting in an elimination of functional lymphocytes bearing receptors for self-antigens (3, 4), the currently available methods do not allow consistent generation of antibodies against self-antigens. In addition, all induced and/or selected antibodies will be directed against epitopes that are not conserved between the two respective species. We hypothesized that the use of t-PA-deficient mice for the production of monoclonal antibodies against mt-PA should not only yield, with a high efficiency, an immune response toward mt-PA, but should also result in the generation of a panel of monoclonal antibodies cross-reacting with t-PA from other species. Immunization with mt-PA and mu-PA indeed caused a significantly higher immune response in gene-inactivated than in wild-type mice.

Out of two fusions using spleen cells isolated from the immunized t-PA^{-/-} mice, 203 stable hybridomas were obtained producing murine monoclonal antibodies against mt-PA, out of which 182 exhibited a cross-reactivity with at least one of the three other PAs tested. Up to 60% cross-reacted with murine, human, and rat t-PA. Of the currently available data regarding monoclonal antibodies raised against ht-PA in wild-type mice, only a few reports mentioned the occurrence of a cross-reactivity with either rt-PA (19) or mt-PA (20). The efficiency of the current approach and its correlation with the extend of homol-

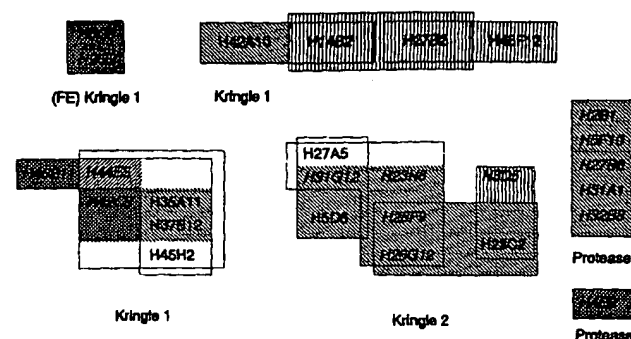


FIG. 1. Epitope mapping of purified antibodies. Cross-reactivities: (□), murine and rat t-PA; (▨), murine, rat and human t-PA; (▩), murine, rat t-PA and vampire bat-PA; (▧), murine, rat, human t-PA and vampire bat-PA. Codes in *italics* represent antibodies with inhibitory properties.

ogy is further illustrated in Table III. Indeed, the higher the percentage of homology, the higher the number of cross-reacting antibodies.

More interestingly, 15% (31 out of 203) of the monoclonal antibodies cross-reacted with mt-PA, rt-PA, ht-PA, and bat-PA. Localization of the domain reactivity of these antibodies revealed that none were directed against the kringle 2 domain. This observation is consistent with the hypothesis, based on its primary structure, that the kringle of bat-PA is more similar to kringle 1 (77% amino acid identity) than to kringle 2 (56% amino acid identity) of human t-PA (21).

From the observation that none of the currently described anti-mt-PA antibodies cross-reacted with mu-PA, one may not conclude that mt-PA and mu-PA would not have any common epitopes since the t-PA^{-/-} mice used had normal u-PA levels (6). Consequently, production of antibodies against epitopes conserved between mt-PA and mu-PA would require the use of "double knocked-out" mice. Because such mice are however severely handicapped (6), this was not attempted.

Monoclonal antibodies can be directed against epitopes involved in the biological or enzymatic activity of proteins, and it is not unlikely that homologous proteins with similar activities have these particular epitopes in common or at least share a significant degree of similarity. In the present study, out of the 203 monoclonal antibodies, 111 inhibited plasminogen activation by mt-PA. However, of the 21 antibodies reacting exclusively with mt-PA, only 6 (29%) exhibited neutralizing properties, whereas out of the 182 monoclonal antibodies cross-reacting with at least one other PA, 105 (58%) were inhibitory. The difference between these ratios is significantly different ($p < 0.02$), suggesting that epitopes required for the biological activity of plasminogen activators are more conserved across species than other regions of the molecule. Three different epitopes (Fig. 1), conserved among all four species, were found to be involved in the plasminogen activation potential of t-PA. Evaluation of their presence in plasminogen activators other than those used in the present study may reveal how far this conservation extends over various other species. In the current study, the inhibiting properties were evaluated using a plasminogen-coupled chromogenic substrate assay, in the presence of fibrin. Using this method, antibodies interfering with the catalytic activity as well as antibodies interfering with plasminogen or fibrin binding are detected. In addition, evaluation of the inhibitory properties of 23 purified antibodies, using a direct assay for t-PA activity, revealed that one cluster of monoclonal antibodies directed against the protease domain (Fig. 1, MA-H2B1, MA-H5F10, MA-H2B6, MA-H31A1, and MA-H32B8), and recognizing an epitope that is conserved

TABLE III
Comparison of interspecies homology and yield of cross-reacting antibodies obtained either from wild-type mice or from t-PA^{-/-} mice

t-PA	Homology (%)		Yield ^a	
	vs. human	vs. murine	n = 20 ^b	n = 203 ^c
Human (FEK ₁ K ₂ P)	100	81	20	158
Murine (FEK ₁ K ₂ P)	81	100	0	203
Rat (FEK ₁ K ₂ P)	81	92	2	175
Vampire bat (FEK ₁ P)	72	72	0	34

^a Number of murine monoclonal antibodies cross-reacting with the respective (t-)PA.

^b Total number of murine monoclonal antibodies raised against human t-PA in wild-type mice.

^c Total number of murine monoclonal antibodies raised against murine t-PA in t-PA^{-/-} mice.

among murine, rat, and human t-PA, also interfered with the catalytic activity of murine as well as human t-PA (data not shown).

The observation that the 38 anti-mu-PA antibodies raised in u-PA^{-/-} mice did not cross-react with hu-PA, even though in line with our previous observations that polyclonal antisera raised in rabbits against hu-PA do not cross-react with mu-PA, should be interpreted with some caution. (a) Only a limited number of monoclonal antibodies could be studied that (b) were obtained out of only one fusion, and (c) no u-PAs from other species were included.

In conclusion, a large panel of murine monoclonal antibodies directed against mt-PA was obtained using t-PA-deficient mice as immunizing target. These antibodies cover a wide range of epitopes localized in different domains of the molecule and conserved among the various species studied. By extrapolation, it is assumed that immunization of any type of gene-inacti-

vated mice with the respective knocked-out proteins could provide a general means to obtain unique monoclonal antibodies against structurally and functionally conserved domains within other protein families.

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ANTIBODY AGAINST METALLOPROTEASE HAVING AGGRECANASE ACTIVITY
NOVEL METALLOPROTEASE HAVING AGGRECANASE ACTIVITY

Cross-References to Related Applications

[01] This is a divisional of Application No. 10/009,332 filed December 10, 2001 (now U.S. Patent No. 6,716,613), which is a national Stage Application filed under §371 of PCT Application No. PCT/JP00/07917 filed November 10, 2000; the above noted prior applications are all hereby incorporated by reference.

Background of the Invention

Technical Field of the Invention

~~[01]~~[02] This invention relates to a novel metalloprotease having an aggrecanase activity and causing joint diseases (to be referred to as "joint disease aggrecanase" hereinafter), a gene coding for this "joint disease aggrecanase", a method for producing the "joint disease aggrecanase", a method for screening a substance capable of inhibiting the aggrecanase activity with the use of the "joint disease aggrecanase", a pharmaceutical composition for inhibiting degradation of proteoglycans, which comprises the substance capable of inhibiting the aggrecanase activity as the active ingredient, and a promoter gene of the "joint disease aggrecanase".

Background Description of the Related Art

~~[02]~~[03] Joint diseases are diseases which show damage and

degeneration of joint cartilage as the main morbid states. Though a disease having the most frequent number of patients among joint diseases is osteoarthritis (OA), analgesic anti-inflammatory drugs and hyaluronic acid preparations are used in the current therapeutic method merely as a symptomatic therapy for the purpose of alleviating pains accompanied by the degeneration of cartilage and the destruction of bone under cartilage, so that it cannot be said that they are exerting sufficient therapeutic effects.

+03+04] Joint cartilage is a tissue mainly composed of type II collagen and aggrecan which is a cartilage-specific proteoglycan, and degradation and degeneration of both of them are observed in the joint diseases. Because of this, it has been considered for a long time that control of the degradation and degeneration of these extracellular matrix components would lead to the treatment of joint diseases, so that attempts have been positively made to identify degradation-concerned proteases (collagenase and aggrecanase) and to screen their inhibitors and develop them as medicaments.

+04+05] As proteases having collagenase activities, matrix metalloproteases (MMP1, MMP8, MMP13, MMP14 and the like) have been identified, and their selective inhibitors have been discovered. However, in spite of the attempts to develop a large number of MMP inhibitors having collagenase inhibition activities as therapeutic drugs for joint diseases including OA and rheumatic arthritis (RA),

MMP inhibitors to be used in these diseases as the indication have not been put on the market. Under such circumstances, attention has been directed toward aggrecanase which selectively degrades aggrecan which is another main constituting component of joint cartilage.

~~[05]~~[06] A joint disease-related role of an enzyme aggrecanase which cleaves aggrecan at the site between Glu³⁷³-Ala³⁷⁴ has been revealed by the reports of Sandy *et al.* and Lohmander *et al.* stating that all of the main digested aggrecan fragments found in the synovial fluid of human arthritis patients were generated by cleaving at the aggrecanase digestion site (Sandy J.D. *et al.*, *J. Clin. Invest.*, 89, 1512 - 1516, 1992; Lohmander L.S. *et al.*, *Arthritis Rheum.*, 36, 1214 - 1222, 1993). On the other hand, it has been known that, in an *in vitro* explant culture system of joint cartilage, degradation of aggrecan firstly occurs by IL-1 induction and then degradation of type II collagen is accelerated (Dingle L.T. *et al.*, *Ann. Rheum. Dis.*, 34, 303 - 311, 1975; Cawston T.E. *et al.*, *Biochem. Biophys. Res. Comm.*, 215, 377 - 385, 1995; Kozaci L.D. *et al.*, *Arthritis Rheum.*, 40, 164 - 174, 1997). It has been reported that the aggrecan degradation takes the precedence of the type II collagen degradation in a mouse arthritis model too (van Meurs J.B. *et al.*, *Arthritis Rheum.*, 42, 1128 - 1139, 1999). These reports suggest a possibility that the type II collagen degradation can be controlled by inhibiting the

preceding aggrecan degradation.

~~{06}~~[07] However, the entity of the aggrecanase which causes joint diseases ("joint disease aggrecanase") has been unclear for long time, though its biochemical properties had been elucidated, namely it is a metalloprotease, it exists in outside of cells, a glycosaminoglycan side chain is concerned in its substrate recognition, its activity is induced by IL-1, TNF and retinoic acid, and the like. Recently, ADAMTS4 (aggrecanase-1: Tortorella M.D. et al., *Science*, 284, 1664 - 1666, 1999) and ADAMTS11 (aggrecanase-2: Abbaszade I. et al., *J. Biol. Chem.*, 274, 23443 - 23450, 1999) have been reported as proteases having an aggrecanase activity. However, it was revealed that they are not the "joint disease aggrecanase", because their gene expression in human OA cartilage is not increased, and their gene expression in an *in vitro* explant culture system of human knee joint cartilage is not induced by IL-1, TNF and retinoic acid which induce the aggrecanase activity that causes joint diseases (Flannery C.R. et al., *Biochem. Biophys. Res. Commun.*, 260, 318 - 322, 1999). As described above, the "joint disease aggrecanase" has not been obtained.

Disclosure-Brief Summary of the Invention

~~{07}~~[08] Under such circumstances, the present inventors have conducted intensive studies and, as a result, succeeded in isolating a gene coding for a novel metalloprotease having the

aggrecanase activity, which is the "joint disease aggrecanase", determining its full-length ORF sequence and thereby achieved production of a recombinant protein.

~~{08}~~[09] Also, a vector comprising this gene, a host cell comprising this vector and a method for producing the novel protein using this host cell were established.

~~{09}~~[10] Also, the inventors have succeeded in providing a screening method which uses this protein and found that a compound selected by carrying out this screening method significantly inhibits the "aggrecanase activity" (namely, the activity of this protein to cleave the extracellular substrate aggrecan selectively at the site between Glu³⁷³-Ala³⁷⁴) and can become a medicament useful in preventing and/or treating joint diseases.

~~{10}~~[11] In addition, a promoter gene of the protein, which is useful in screening a medicament for preventing and/or treating joint diseases was isolated, resulting in accomplishment of the present invention.

~~{11}~~[12] Accordingly, the invention relates to:

~~{12}~~[13] [1] a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease,

~~{13}~~[14] [2] a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 1st position to the

583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease,

~~[14]~~[15] [3] a metalloprotease having an aggrecanase activity, which consists of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 687th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 687th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease,

~~[15]~~[16] [4] a gene which encodes the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or an amino acid sequence of an equivalent of the metalloprotease,

~~[16]~~[17] [5] a vector which comprises the gene described in [4],

~~[17]~~[18] [6] a host cell which comprises the vector described in [5],

~~[18]~~[19] [7] a method for producing the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or an equivalent of the metalloprotease, which comprises using the host cell described in [6],

~~{19}~~[20] [8] an antibody against the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or an equivalent of the metalloprotease,

~~{20}~~[21] [9] a method for screening a substance capable of inhibiting an aggrecanase activity of the metalloprotease, which comprises allowing the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or an equivalent of the metalloprotease to contact with a compound to be tested,

~~{21}~~[22] [10] a pharmaceutical composition for inhibiting degradation of proteoglycans, which comprises a substance capable of inhibiting the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or an equivalent of the metalloprotease, as an active ingredient, and

~~{22}~~[23] [11] a gene represented by SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31, or an equivalent of the gene.

~~{23}~~[24] The invention also relates to the use of a substance capable of inhibiting an aggrecanase activity of the metalloprotease having an aggrecanase activity described in any one of [1] to [3] or of an equivalent of the metalloprotease, in producing a medicament for inhibiting degradation of proteoglycans.

~~{24}~~[25] The invention also relates to the use of a substance capable of inhibiting the metalloprotease having an aggrecanase activity or an equivalent of the metalloprotease, which is obtainable by the screening method described in [9], in treating joint diseases.

~~[25]~~[26] The invention also relates to a method for screening a substance capable of modifying a promoter activity of the gene described in [11], which uses this gene.

Brief Description of the Drawings

Mode for Carrying Out the Invention

[27] Fig. 1 is a photograph showing a result of the expression of MDTS6TSP1 in an animal cell strain using an ECL western blotting detection system, obtained in Example 6.

[28] Fig. 2 is a photograph showing a result of the detection of the activity of MDTS6TSP1 to degrade a recombinant aggrecan G1G2 using an ECL western blotting detection system, obtained in Example 7-2.

[29] Fig. 3 is a photograph showing a result of the analysis of a recombinant aggrecan G1G2 degraded with MDTS6TSP1, by an anti-aggrecanase neoepitope antibody, using a western blotting detection system, obtained in Example 7-3.

[30] Fig. 4 is an electrophoresis pattern photograph showing a result of the examination of MDTS6 mRNA expression induction by IL-1 β , obtained in Example 8.

[31] Fig. 5 is a photograph showing a result of the detection of degradation of natural type aggrecan by MDTS6 protein, by an anti-aggrecanase neoepitope antibody, using a western blotting detection system, obtained in Example 9-2.

[32] Fig. 6 is a graph showing a result of the detection of release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid and IL-1 β , obtained in Example 11-2.

[33] Fig. 7 is an electrophoresis pattern photograph showing a result of the analysis of changes in gene expression of MDS6 by RT-PCR when rabbit knee joint primary culture cells are treated with all-trans retinoic acid and IL-1 β , obtained in Example 11-3.

[34] Fig. 8 is a graph showing that degradation and release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid are inhibited by the compound A and compound B, obtained in Example 12.

Detailed Description of the Invention

~~[26]~~[35] The following describes the terms used in the invention.

The term "aggrecanase" as used herein means a metalloprotease which has a zinc binding consensus sequence (HEXXH) and also has an activity to cleave aggrecan existing in joint cartilage selectively at the site between Glu³⁷³-Ala³⁷⁴, namely the "aggrecanase activity". Also, unless otherwise noted, the "aggrecanase" is referred to as "protein".

~~[27]~~[36] The "joint disease aggrecanase" of the invention is any of a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or

an equivalent of the metalloprotease.

~~{28}~~[37] Also, the "joint disease aggrecanase" of the invention is preferably a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease.

~~{29}~~[38] More preferably, it is a metalloprotease having an aggrecanase activity, which consists of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 687th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 687th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease.

~~{30}~~[39] Regarding the "equivalent of the metalloprotease", (1) in the case of an equivalent of the metalloprotease comprising an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it

is a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in the amino acid sequence of from the 213th position to the 583rd position, and which has the aggrecanase activity, (2) in the case of an equivalent of the metalloprotease comprising an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it is a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in the amino acid sequence of from the 1st position to the 583rd position, and which has the aggrecanase activity, or (3) in the case of an equivalent of the metalloprotease consisting of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 687th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 687th position of the amino acid

sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it is a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in respective sequences, and which has the aggrecanase activity.

~~{31}~~[40] Origin of the "joint disease aggrecanase" of the invention is not limited to human. For example, it includes a metalloprotease having the aggrecanase activity which is originated from an organism other than human (e.g., mouse, rat, hamster and dog) and cause joint diseases. Also included is a protein artificially modified by a genetic engineering means based on the sequence of "joint disease aggrecanase" described in SEQ ID NO:1.

~~{32}~~[41] Also, the gene coding for the "joint disease aggrecanase" of the invention is any gene which encodes the "joint disease aggrecanase", namely a gene which encodes a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease.

~~{33}~~[42] Also, the gene coding for the "joint disease aggrecanase" of

the invention may be any gene coding for a metalloprotease having an aggrecanase activity, which comprises an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease.

~~[34]~~[43] In addition, it may be any gene coding for a metalloprotease having an aggrecanase activity, which consists of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 687th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 687th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, or an equivalent of the metalloprotease.

~~[35]~~[44] Regarding the "gene coding for an equivalent of the metalloprotease", (1) in the case of a gene coding for an equivalent of the metalloprotease comprising an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it is a gene

coding for a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in the amino acid sequence of from the 213th position to the 583rd position, and which has the aggrecanase activity, (2) in the case of a gene coding for an equivalent of the metalloprotease comprising an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it is a gene coding for a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in the amino acid sequence of from the 1st position to the 583rd position, and which has the aggrecanase activity, or (3) in the case of a gene coding for an equivalent of the metalloprotease consisting of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 687th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 1st position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the 213th position to the 950th position of the amino acid sequence represented by SEQ ID NO:1, an amino acid sequence of from the

213th position to the 687th position of the amino acid sequence represented by SEQ ID NO:1 or an amino acid sequence of from the 213th position to the 583rd position of the amino acid sequence represented by SEQ ID NO:1, it is a gene coding for a metalloprotease in which one to several amino acid residues (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in respective sequences, and which has the aggrecanase activity.

~~[36]~~[45] The gene coding for the "joint disease aggrecanase" of the invention is preferably a gene consisting from the 1st position to the 1749th position, from the 1st position to the 2061st position, from the 1st position to the 2850th position, from the 637th position to the 1749th position, from the 637th position to the 2061st position or from the 637th position to the 2850th position, of the nucleotide sequence described in SEQ ID NO:2, more preferably a gene consisting from the 637th position to the 1749th position, from the 637th position to the 2061st position or from the 637th position to the 2850th position, of the nucleotide sequence described in SEQ ID NO:2.

~~[37]~~[46] The promoter gene of the invention is preferably a gene having a nucleotide sequence described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31. The "equivalent of the gene described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31" is a gene in which one to

several bases (preferably from 1 to 10, more preferably from 1 to 5) are substituted, deleted and/or inserted at one to several positions (preferably from 1 to 10, more preferably from 1 to 5) in the nucleotide sequence described in SEQ ID NO:24, 25, 26, 27, 28, 29, 30 or 31, and which has a "joint disease aggrecanase" promoter activity. The term "promoter activity" means an activity which acts as the initiation region for transcribing information of DNA chains to RNA chains.

[38][47] According to a result of BLAST (basic local alignment search tool) (S.F. Altschul et al., (1990), *J. Mol. Biol.*, 215, 403 - 410) retrieving of GENBANK and SwissProt, the amino acid sequence (SEQ ID NO:1) (950 amino acids) of MDTS6 as one of the "joint disease aggrecanase" of the invention and the nucleotide sequence (SEQ ID NO:2) (2853 base pairs) which encodes this amino acid sequence are novel. When homology of the amino acid sequence with the ADAMTS4 and ADAMTS11 described in the foregoing was examined, its sequence similarity was 50% or less.

[39][48] A metalloprotease having an aggrecanase activity, which has high homology with the metalloprotease having the amino acid sequence represented by SEQ ID NO:1, is also included in the "joint disease aggrecanase" of the invention. The high homology metalloprotease having an aggrecanase activity is a metalloprotease having an aggrecanase activity which shows at least 70% or more, preferably 80% or more, more preferably 90% or

more, most preferably 95% or more, particularly preferably 99% or more, of sequence homology with the amino acid sequence represented by SEQ ID NO:1. The homology can be specified using the aforementioned BLAST retrieving algorithm.

~~[40]~~[49] In addition, the "joint disease aggrecanase" of the invention can be used for the screening of a substance which inhibits the aggrecanase activity that causes joint diseases. The substance which inhibits the aggrecanase activity is useful as a composition for inhibiting degradation of proteoglycans.

~~[41]~~[50] In addition, the promoter gene of the "joint disease aggrecanase" of the invention is worthy of notice in that it can be used for the screening of a substance which inhibits the promoter activity. The term "a substance which inhibits the promoter activity" as used herein means a substance which inhibits expression of the "joint disease aggrecanase" by inhibiting action of the promoter. A method for screening a substance capable of inhibiting promoter activity, which uses the promoter gene of the aggrecanase, and use of the substance capable of inhibiting the promoter activity for preventing and/or treating joint diseases are also included in the invention. Furthermore, the "joint disease aggrecanase" promoter gene exists in two or more mutant forms, namely genetic polymorphism. Thus, it can be used for the analysis of correlation between the genetic polymorphism and diseases in which concern of the aggrecanase is considered so that,

including joint diseases, as a result, there is a possibility that it can be used as a marker for gene diagnosis.

~~[42]~~[51] Regarding the gene coding for the "joint disease aggrecanase" of the invention, the vector of the invention, the host cell of the invention, the method of the invention for producing the "joint disease aggrecanase", the method of the invention for detecting the aggrecanase activity of the "joint disease aggrecanase", the method of the invention for producing an antibody which reacts with the "joint disease aggrecanase", the method of the invention for screening a substance which inhibits the aggrecanase activity of the "joint disease aggrecanase", the method of the invention for detecting the promoter activity and the method of the invention for screening a substance which modifies the promoter activity are described in the following items 1) to 7). All of the items described in 1) to 7) are included in the invention. In the following items 1) to 7), the "joint disease aggrecanase" is described as "protein".

~~[43]~~[52] 1) Production method of protein gene

~~[44]~~[53] a) First production method - a method which uses PCR

~~[45]~~[54] A mRNA sample is extracted from a human cell or tissue having the ability to produce the novel protein of the invention. Next, using this mRNA as the template, two primers interposing the mRNA or a part of mRNA of the novel protein are prepared. Full-length cDNA or a part thereof corresponding to the novel protein

can be obtained by modifying denature temperature, denaturing agent adding condition and the like and carrying out a reverse transcriptase-polymerase chain reaction (to be referred to as RT-PCR hereinafter) suited for a respective protein comprising a part of the amino acid sequence represented by SEQ ID NO:1 of the invention. Alternatively, full-length cDNA or a part thereof corresponding to the novel protein can be obtained by carrying out a polymerase chain reaction (to be referred to as RT-PCR hereinafter), by using cDNA prepared using reverse transcriptase from mRNA which is extracted from a human cell or tissue having the ability to produce the novel protein of the invention, or a commercially available cDNA preparation derived from a human cell or tissue, as the template. Thereafter, the novel protein can be produced by integrating the thus obtained full-length cDNA or a part thereof corresponding to the novel protein into an appropriate expression vector and expressing it in a host cell.

[46][55] Firstly, mRNA comprising a sequence coding for the protease is extracted from a human cell or tissue having the ability to produce the novel protein of the invention by a known method. As the extraction method, a guanidine thiocyanate hot phenol method, a guanidine thiocyanate-guanidine hydrochloride method and the like can be exemplified, but a guanidine thiocyanate cesium chloride method can be preferably cited. The cell or tissue having the ability to produce this protease can be specified by,

e.g., northern blot technique using a gene or a part thereof having a nucleotide sequence coding for the protease or western blot technique using an antibody specific for the protease.

~~[47]~~[56] Purification of mRNA can be carried out in accordance with a usual method, for example, it can be purified by binding it to an oligo(dT) cellulose column and then eluting it. Alternatively, a commercially available extracted and purified mRNA may be used without extracting the mRNA.

~~[48]~~[57] Subsequently, single-strand cDNA is synthesized by carrying out reverse transcriptase reaction of the purified mRNA in the presence of random primers, oligo(dT) primers or custom-synthesized primers. Using two primers interposing a part of the gene of interest, the thus obtained single-strand cDNA is subjected to PCR to amplify the novel protein DNA of interest. Alternatively, a commercially available cDNA preparation may be used without synthesizing the cDNA. The thus obtained DNA is fractionated by a means such as agarose gel electrophoresis or the like. If desired, a DNA fragment of interest can be obtained by digesting this DNA with restriction enzymes and the like and then ligating the digested fragments.

~~[49]~~[58] b) Second production method

~~[50]~~[59] In addition to the above production method, the gene of the invention can be produced using conventional genetic engineering techniques. Firstly, single-strand cDNA is synthesized using

reverse transcriptase and using the mRNA obtained by the above method as the template and then double-strand cDNA is synthesized from this single-strand cDNA. As the method, the S1 nuclease method (Efstratiadis, A. et al., *Cell*, 7, 279 - 288, 1976), Land method (Land, H. et al., *Nucleic Acids Res.*, 9, 2251 - 2266, 1981), O. Joon Yoo method (Yoo, O.J. et al., *Proc. Natl. Acad. Sci. USA*, 79, 1049 - 1053, 1983), the Okayama-Berg method (Okayama, H. and Berg, P., *Mol. Cell. Biol.*, 2, 161 - 170, 1982) and the like can be exemplified.

~~[51]~~[60] Next, an *Escherichia coli* strain such as DH5 α strain, HB101 strain, JM109 strain or the like is transformed by introducing a recombinant plasmid obtained by the aforementioned method, and a resulting recombinant can be selected using the resistance for a drug such as tetracycline, ampicillin, kanamycin or the like as a marker. Transformation of a host cell, for example, when the host cell is *E. coli*, can be carried out by Hanahan's method (Hanahan, D.J., *Mol. Biol.*, 166, 557 - 580, 1983), namely, by adding the recombinant DNA to competent cells prepared in the coexistence of CaCl_2 and MgCl_2 or RbCl . As a matter of course, commercially available competent cells can also be used. In this connection, in addition to a plasmid, a phage vector such as a lambda system can also be used as a vector.

~~[52]~~[61] Regarding the method for selecting DNA of the novel protein of interest from the thus obtained transformants, various methods

shown below can for example be employed.

~~+53+~~[62] (i) A screening method which uses a synthetic oligonucleotide probe

~~+54+~~[63] An oligonucleotide corresponding to whole or a part of the novel protein of the invention is synthesized (in this case, it may be either a nucleotide sequence derived by using the codon usage or a combination of two or more possible nucleotide sequences, and in the latter case, the number of their kinds can be reduced by including inosine), this is hybridized as a probe (after labeling with ^{32}P or ^{33}P) with a nitrocellulose filter or nylon filter on which DNA samples of the transformants are denatured and immobilized, and then the thus obtained positive strains are screened and selected.

~~+55+~~[64] (ii) A screening method which uses a probe prepared by polymerase chain reaction

~~+56+~~[65] Oligonucleotides of a sense primer and an antisense primer corresponding to a part of the novel protein of the invention are synthesized and polymerase chain reaction (Saiki, R.K. et al., *Science*, 239, 487 - 491, 1988) is carried out using these primers, thereby effecting amplification of a DNA fragment coding for whole or a part of the novel protein of interest. As the template DNA to be used, cDNA synthesized by reverse transcription reaction from mRNA of cells producing the novel protein or genomic DNA can be used. The thus prepared DNA fragment is labeled with ^{32}P or ^{33}P

and used as the probe to carry out colony hybridization or plaque hybridization to select the clone of interest.

~~+57+~~[66] (iii) A screening method in which the novel protein is produced by other animal cells

~~+58+~~[67] A transformant is cultured to amplify a gene, an animal cell is transfected with the gene (in this case, the vector may be either an autonomously replicating plasmid comprising a transcription promoter region or a plasmid which can be integrated into chromosome of the animal cell), and the protein encoded by the gene is produced in the extracellular moiety. By detecting the novel protein using an antibody specific for the novel protein of the invention, a strain comprising cDNA which encodes the novel protein of interest is selected from the original transformants.

~~+59+~~[68] (iv) A selection method which uses an antibody specific for the novel protein of the invention

~~+60+~~[69] By integrating cDNA into an expression vector in advance, proteins are produced in culture supernatants, inside the cells or on the surface of cells of transformants, and the strain of interest is selected by detecting the novel protein producing strain of interest using an antibody specific for the novel protein of the invention and a secondary antibody against this antibody.

~~+61+~~[70] (v) A method which uses a selective hybridization-translation system

~~[62]~~[71] Samples of cDNA obtained from transformants are blotted on a nitrocellulose filter or the like, mRNA prepared from the novel protein producing cells of the invention is hybridized therewith, and then the mRNA hybridized to the cDNA is dissociated and recovered. The thus recovered mRNA samples are translated into proteins in a protein translation system, e.g., a system in which they are injected into oocyte of *Xenopus* or a cell free system such as rabbit reticulocyte lysate, wheat germ or the like. The strain of interest is selected by detecting it using an antibody against the novel protein of the invention.

~~[63]~~[72] The method for collecting DNA coding for the novel protein of the invention from the thus obtained transformant of interest can be carried out in accordance with gene manipulation experiment manuals such as of a known method (Sambrook, J. et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989) and the like. For example, it can be achieved by separating a fraction corresponding to plasmid DNA from cells and then cutting out the cDNA region from the plasmid DNA.

~~[64]~~[73] c) Third production method

~~[65]~~[74] The novel protein gene of the invention can also be produced by connecting DNA fragments produced by a chemical synthesis method. Each DNA can be synthesized using a DNA synthesizing machine [e.g., Oligo 1000M DNA Synthesizer (mfd. by Beckman), 394 DNA/RNA Synthesizer (mfd. by Applied Biosystems) or the like].

{66}[75] d) Fourth production method

{67}[76] The novel protein gene of the invention can also be produced based on the information on the novel protein, for example, by chemical synthesis of nucleic acids in accordance with a conventional method such as phosphite triester method (Hunkapiller, M. et al., *Nature*, 10, 105 - 111, 1984) or the like. In this connection, codons for desired amino acids are well known, can be selected optionally and can be determined in accordance with a conventional method (Crantham, R. et al., *Nucleic Acids Res.*, 9, r43 - r74, 1981), taking codon usage of the host to be used into consideration. In addition, partial modification of codons of these nucleotide sequences can be carried out in the usual way in accordance with the site specific mutagenesis (Mark, D.F. et al., *Proc. Natl. Acad. Sci. USA*, 81, 5662 - 5666, 1984) or the like which uses primers comprised of synthetic oligonucleotides which encode the desired modification.

{68}[77] Determination of sequences of DNA obtained by the above methods a) to d) can be carried out for example by Maxam-Gilbert chemical modification method (Maxam, A.M. and Gilbert, W., "Methods in Enzymology", 65, 499 - 559, 1980), dideoxy nucleotide chain termination method (Messing, J. and Vieira, J., *Gene*, 19, 269 - 276, 1982) and the like.

{69}[78] 2) Methods for the production of the vector of the invention, the host cell of the invention and the recombinant protein of the

invention

~~[70]~~[79] The thus isolated fragment containing the gene coding for the novel protein of the invention can be transformed into eucaryotic or procaryotic host cells by again integrating it into an appropriate vector DNA. In addition, it is possible to express the gene in respective host cells by introducing an appropriate promoter and a sequence concerned in the gene expression into these vectors.

~~[71]~~[80] For example, the eucaryotic host cells include cells of a vertebrate, an insect, yeast and the like, and COS cell as a monkey cell (Gluzman, Y., *Cell*, 23, 175 - 182, 1981), a dihydrofolate reductase deficient strain of Chinese hamster ovary cell (CHO) (Urlaub, G. and Chasin, L.A., *Proc. Natl. Acad. Sci. USA*, 77, 4216 - 4220, 1980), human fetal kidney-derived HEK293 cell, 293-EBNA cell in which Epstein-Barr virus EBNA-1 gene is introduced into the same cell (mfd. by Invitrogen) and the like are frequently used as the vertebrate cells, though limited thereto.

~~[72]~~[81] As the expression vector for vertebrate cells, a vector having a promoter, a RNA splicing site, a polyadenylation site, a transcription termination sequence and the like generally positioned upstream of the gene to be expressed can be used, and it may further have a replication origin as occasion demands. Examples of the expression vector include pSV2dhfr having SV40

early promoter (Subramani, S. et al., *Mol. Cell. Biol.*, 1, 854 - 864, 1981), pEF-BOS having human elongation factor promoter (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18, 5322, 1990), pCEP4 having cytomegalovirus promoter (mfd. by Invitrogen) and the like, though not limited thereto.

[73][82] In the case of the use of COS cell as the host cell, an expression vector which has SV40 replication origin, can perform autonomous replication in COS cell and has a transcription promoter, a transcription termination signal and an RNA splicing site can be used, and its examples include pME18S (Maruyama, K. and Takebe, Y., *Med. Immunol.*, 20, 27 - 32, 1990), pEF-BOS (Mizushima, S. and Nagata, S., *Nucleic Acids Res.*, 18, 5322, 1990), pCDM8 (Seed, B., *Nature*, 329, 840 - 842, 1987) and the like. The expression vector can be incorporated into COS cell by a DEAE-dextran method (Luthman, H. and Magnusson, G., *Nucleic Acids Res.*, 11, 1295 - 1308, 1983), a calcium phosphate-DNA co-precipitation method (Graham, F.L. and van der Ed, A.J., *Virology*, 52, 456 - 457, 1973), a method which uses FuGENE™6 Transfection Reagent (mfd. by Boehringer Mannheim), an electroporation method (Neumann, E. et al., *EMBO J.*, 1, 841 - 845, 1982) and the like, thus enabling to obtain a desired transformant cell.

[74][83] Also, when CHO cell is used as the host cell, a transformant cell which can stably produce the novel protein can be obtained by co-transfecting a vector capable of expressing neo gene which

functions as a G418 resistance marker, such as pRSVneo (Sambrook, J. et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989), pSV2-neo (Southern, P.J. and Berg, P., *J. Mol. Appl. Genet.*, 1, 327 - 341, 1982) or the like, together with an expression vector and then selecting a G418-resistant colony. Also, when 293-EBNA cell is used as the host cell, a desired transformant cell can be obtained using an expression vector which has Epstein-Barr virus replication origin and can perform autonomous replication in the 293-EBNA cell, such as pCEP4 (mfd. by Invitrogen) or the like.

[75][84] The thus obtained transformant cell of interest can be cultured in accordance with a conventional method, and the novel protein of the invention is produced in extracellular moiety by this culturing. As the medium to be used in the culturing, various conventionally used media can be optionally selected depending on the host cell employed. In the case of, for example, the COS cell, a medium such as a RPMI-1640 medium, Dulbecco's modified Eagle's minimum essential medium (DMEM) or the like which may be supplemented, as occasion demands, with a serum component such as fetal bovine serum (FBS) or the like may be used. Also, in the case of the 293-EBNA cell, a medium such as Dulbecco's modified Eagle's minimum essential medium (DMEM) or the like supplemented with a serum component such as fetal bovine serum (FBS) or the like and further supplemented with G418 may be used.

{76}[85] The novel protein of the invention thus produced in the extracellular moiety of the transformant cell can be separated and purified by various known separation techniques making use of physical characteristics, biochemical characteristics and the like of the novel protein. Illustrative examples of such techniques include treatment of a culture broth containing the novel protein with a usual protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, high performance liquid chromatography (HPLC) and the like, dialysis and combinations thereof.

{77}[86] When the novel protein of the invention is expressed after its in frame fusion with a marker sequence, expression verification, purification and the like of the novel protein become possible. Examples of the marker sequence include FLAG epitope, Hexa-Histidine tag, Hemagglutinin tag, myc epitope and the like. Also, when a specific amino acid sequence recognizable by proteases such as enterokinase, factor Xa, thrombin and the like is inserted between a marker sequence and the novel protein, the marker sequence moiety can be cut off and removed by these proteases.

{78}[87] 3) Method for detecting the aggrecanase activity of the protein of the invention

~~[79]~~[88] The aggrecanase activity of the protein of the invention can be detected by mixing the joint disease aggrecanase of the invention with each of the substrates described below in an appropriate buffer solution, allowing them to react with each other and then detecting the reaction product by a method suited for each substrate.

~~[80]~~[89] As the substrate, aggrecan purified from a cartilage or tissue of human or other animal, aggrecan obtained by genetic recombination, commercially available aggrecan (mfd. by Seikagaku Kogyo) or a partial protein thereof can be used. The aggrecanase activity can be measured by allowing these substrates to react with a cell or tissue culture broth, a cell or tissue extract or a (partially) purified sample containing a protease to be tested, and then detecting a fragment cleaved off at the site between Glu³⁷³-Ala³⁷⁴. The fragment cleaved off at the site between Glu³⁷³-Ala³⁷⁴ can be detected by a method in which an N-terminal sequence or a C-terminal sequence of the digested fragment is determined in accordance with a conventional method, or more conveniently, by an immunological method such as an ELISA (enzyme-linked immunosorbent assay) which uses an anti-neoepitope antibody capable of specifically recognizing C-terminal NITGE³⁷³ and N-terminal ³⁷⁴ARGSV generated by the cutting between Glu³⁷³-Ala³⁷⁴, a western blotting or the like. Preferably, it can be carried out by the methods described in Examples 7 and 9.

{81}[90] 4) Method for preparing antibody which reacts with the novel protein of the invention

{82}[91] The antibody which reacts with the novel protein of the invention, such as a polyclonal antibody or a monoclonal antibody, can be obtained by directly administering the novel protein or a fragment of the novel protein to various animals. It can also be obtained by a DNA vaccine method (Raz, E. et al., *Proc. Natl. Acad. Sci. USA*, 91, 9519 - 9523, 1994; Donnelly, J.J. et al., *J. Infect. Dis.*, 173, 314 - 320, 1996) using a plasmid into which a gene coding for the novel protein of the invention is introduced.

{83}[92] A polyclonal antibody is produced from a serum or egg of an animal such as rabbit, rat, goat, domestic fowl or the like which is sensitized by immunizing the animal with the novel protein or a fragment thereof emulsified in an appropriate adjuvant such as complete Freund's adjuvant by its peritoneal, subcutaneous, intravenous or the like injection. The thus produced polyclonal antibody can be separated and purified by usual protein isolation and purification techniques, and examples of the usual protein isolation and purification techniques include centrifugation, dialysis, salting out with ammonium sulfate and a chromatography using DEAE-cellulose, hydroxyapatite, protein A agarose or the like.

{84}[93] A monoclonal antibody can be produced easily by those skilled in the art by the cell fusion method of Kohler and

Milstein (Kohler, G. and Milstein, C., *Nature*, 256, 495 - 497, 1975).

~~[85]~~[94] That is, mouse is immunized by emulsifying the novel protein of the invention or a fragment thereof in an appropriate adjuvant such as complete Freund's adjuvant and inoculating the emulsion several times at intervals of a few weeks by its peritoneal, subcutaneous, intravenous or the like injection. After the final immunization, spleen cells are taken out and fused with myeloma cells to prepare hybridomas.

~~[86]~~[95] As the myeloma cells for obtaining hybridomas, a myeloma cell having a marker (e.g., hypoxanthine-guanine phosphoribosyl transferase deletion, thymidine kinase deletion or the like), such as a mouse myeloma cell strain P3X63Ag8.U1, is used. Also, polyethylene glycol is used as the fusing agent. As the medium for preparing hybridomas, Eagle's minimum essential medium, Dulbecco's minimum essential medium, RPMI1640 or the like usually used medium is used by optionally supplementing it with 10 to 30% fetal bovine serum. The fused strains are selected by the HAT selection method. Screening of hybridomas is carried out using culture supernatants by ELISA, immunological tissue staining or the like well known method or by the aforementioned screening method, and a clone of hybridoma which secretes the antibody of interest is selected. Also, monoclonal nature of the hybridoma is confirmed by repeating subcloning by limiting dilution. By

culturing the thus obtained hybridoma in a medium for several days or in the abdominal cavity of a pristane-pretreated BALB/c mouse for 10 to 20 days, the antibody is produced in a purification-possible amount. The thus produced monoclonal antibody can be separated and purified from the culture supernatant or ascitic fluid by usual protein isolation purification techniques.

~~{87}~~[96] Active antibody fragments containing a part of the antibody, such as $F(ab')_2$, Fab, Fab' and Fv, can be obtained by digesting the thus separated and purified antibody with a proteolytic enzyme such as pepsin, papain or the like in the conventional way and then separating and purifying the fragments by usual protein isolation purification techniques.

~~{88}~~[97] Furthermore, it is possible to obtain the antibody which reacts with the novel protein of the invention as single chain Fv or Fab by the methods of Clackson *et al.* and Zebedee *et al.* (Clackson, T. *et al.*, *Nature*, 352, 624 - 628, 1991; Zebedee, S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 3175 - 3179, 1992). In addition, it is possible to obtain a human antibody by immunizing a transgenic mouse in which a mouse antibody gene is replaced by a human antibody gene (Lonberg, N. *et al.*, *Nature*, 368, 856 - 859, 1994).

~~{89}~~[98] 5) Method for screening a substance which inhibits the aggrecanase activity of the "joint disease aggrecanase" of the invention

~~{90}~~[99] This can be screened by a similar method of the aggrecanase activity detection method described in 3). Also, the ELISA or the like method exemplified in Example 10-2 can be used, in which added aggrecan, recombinant aggrecan, commercially available aggrecan or a partial protein thereof which disappears or decreases by its degradation when allowed to react with the novel protein of the invention is measured using an antibody which specifically recognizes polypeptides of the N-terminal side and C-terminal side moieties of the region cleaved off with aggrecanase. Also useful is a method in which the novel protein of the invention is allowed to react with a recombinant aggrecan in which FLAG tag is added to the N-terminal, and His tag to the C-terminal, as exemplified in Example 7-1, and the added recombinant aggrecan disappeared or decreased by its degradation is measured by ELISA or the like method using an anti-FLAG tag and anti-His tag antibodies. The tags in this case are not limited to FLAG tag and His tag, and the recombinant aggrecan is not limited to Example 7-1 and may be a partial protein or modified protein of aggrecan which is cleaved off at the aggrecanase digesting site by this protein. Regarding the substance to be tested for its aggrecanase activity, compounds or peptides which are generally known to have metalloprotease inhibition activity but their activities to inhibit the aggrecanase activity of the novel protein are unclear, or various known compounds and peptides, compounds synthesized

using combinatorial chemistry techniques (Terrett, N.K. et al., *Tetrahedron*, 51, 8135 - 8137, 1995) or general synthesis techniques and random peptides prepared by applying a phage display method (Felici, F. et al., *J. Mol. Biol.*, 222, 301 - 310, 1991) and the like, can be used as the substance to be tested. In addition, extracts and culture supernatants of microorganisms, natural components derived from plants and marine organisms, animal tissue extracts and the like are also become objects of the screening. Or possibly, compounds or peptides prepared by chemically or biologically structure-modified from compounds or peptides selected by the screening method of the invention can also be used.

{91}[100] For the screening of substances which inhibit the aggrecanase activity of the novel protein of the invention (compounds, peptides, antibodies and antibody fragments), any substance which becomes the substrate of the novel protein of the invention or of a partial peptide thereof can be used, and the substrates described in the aforementioned item 3) are desirable.

{92}[101] 6) Method for detecting degradation and release of proteoglycan

{93}[102] A method exemplified in Example 11-2 in which $^{35}\text{SO}_4^{2-}$ is used as a tracer, a method in which a proteoglycan antibody is used, a method in which degraded fragments are detected by gel filtration (Methods in Cartilage Research, Academic Press, 1990; Joint

Cartilage Degradation, Marcel Dekker, Inc., 1993), a colorimetric method (Goldberg R.L. and Kolibas L.M., *Connect. Tissue Res.*, 24, 265 - 275, 1990) which uses 1,9-dimethylmethylene blue (DMMB) and the like are used for the detection and measurement of the degradation and release of cartilage proteoglycan, though not limited thereto.

~~[94]~~[103] 7) Method for screening a substance which inhibits promoter activity of the invention

~~[95]~~[104] In screening a substance which inhibits the promoter activity of the invention, a method in which a reporter gene plasmid containing the sequences shown in Example 13 (SEQ ID NOs:24 and 31) and partial sequences thereof is used is convenient as the method for detecting the promoter activity. The reporter gene means a gene coding for a protein which can be determined by usual means (e.g., determination methods well known to those skilled in the art such as measurement of enzyme activities and the like), and chloramphenicol acetyltransferase, luciferase, β -galactosidase and alkaline phosphatase genes are frequently used, though not limited thereto. Regarding a vector for constructing a reporter gene plasmid, there is no limitation and commercially available plasmid vectors such as pGV-B2 (mfd. by Toyo Ink), pSEAP2-Basic (mfd. by Clontech) and the like can be used. By constructing a reporter gene plasmid in which the sequence is inserted in the forward direction into upstream of the reporter

gene of these vectors and measuring amount of the reporter protein expressed in cells transformed with this plasmid, by a method suited for respective case, the presence and strength of the promoter activity of the sequence can be known, and action of a substance to be tested upon this promoter activity can be detected by adding the substance to be tested to a culture broth of the transformed cells.

~~496~~[105] For the screening of substances which inhibit the promoter activity possessed by the sequence of the Sequence ID No. of the invention and a partial sequence thereof (compounds, peptides, antibodies and antibody fragments), a method similar to the aforementioned promoter activity detection method can be used. Regarding the substance to be tested, compounds or peptides which are generally known to have promoter inhibition activity but their activities to inhibit the promoter activity possessed by the sequences of SEQ ID NOs:24 and 31 and partial sequences thereof are unclear or various known compounds and peptides, compounds synthesized using combinatorial chemistry techniques (Terrett, N.K. *et al.*, *Tetrahedron*, 51, 8135 - 8137, 1995) or general synthesis techniques and random peptides, antibodies and antibody fragments prepared by applying a phage display method (Felici, F. *et al.*, *J. Mol. Biol.*, 222, 301 - 310, 1991) can be used. In addition, extracts and culture supernatants of microorganisms, natural components derived from plants and marine organisms, animal tissue

extracts and the like are also become the object of the screening. Or possibly, compounds or peptides prepared by chemically or biologically structure-modified from compounds or peptides selected by the screening method of the invention can also be used.

~~[97]~~[106] A medicament which comprises as the active ingredient a substance which inhibits the aggrecanase activity of the "joint disease aggrecanase" and is selected by the aforementioned screening method (a compound, peptide, antibody or antibody fragment) is included in the invention, and a pharmaceutical composition for inhibiting degradation of proteoglycans is particularly desirable as the medicament. Examples of the substance which significantly inhibits activity of the "joint disease aggrecanase" include N^{α} -[2-(1-hydroxycarbamoyl-2-sulfanylethyl)-4-methylpentanoyl]-N,O-dimethyltyrosineamide (to be referred to as compound A hereinafter), N^{α} -[2-(1-hydroxycarbamoyl-2-sulfanylethyl)-4-methylpentanoyl]-N-methylphenylalanineamide (to be referred to as compound B hereinafter), N^{α} -[2-(1-hydroxycarbamoyl-2-phenylsulfanylethyl)-4-methylpentanoyl]-N,O-dimethyltyrosineamide (to be referred to as compound C hereinafter), N^{α} -[2-(1-hydroxycarbamoyl-2-methylsulfanylethyl)-4-methylpentanoyl]-N,O-dimethyltyrosineamide (to be referred to as compound D hereinafter) and the like selected by the screening system shown in Example 10-2. The compound A, compound B, compound C and compound D are compounds included in the claims of

WO 90/05719, but not only medicaments comprising these compounds as the active ingredient but also all medicaments which comprises substances capable of significantly inhibiting the aggrecanase activity of the "joint disease aggrecanase" are included in the invention. In this connection, the compound A, compound B, compound C and compound D are compounds can be synthesized in the same manner as the compounds disclosed in WO 90/05719 in accordance with the production methods disclosed in WO 90/05719.

~~{98}~~[107] The medicament comprising a substance (a compound, peptide, antibody or antibody fragment) which significantly inhibits the aggrecanase activity of the "joint disease aggrecanase" of the invention as the active ingredient can be prepared using carriers, fillers and other additives usually used for their preparation, in response to each type of the active ingredient.

~~{99}~~[108] Examples of its administration include oral administration using tablets, pills, capsules, granules, fine subtilaes, powders, oral solutions and the like and parenteral administration using intravenous, intramuscular, intraarticular and the like injections, suppositories, percutaneous preparations, transmucosal preparations and the like. Particularly in the case of peptides which are digested in the stomach, parenteral administration such as intravenous injection or the like is desirable.

~~{100}~~[109] In the solid composition for use in the oral administration according to the present invention, one or more active substances

are mixed with at least one inert diluent such as lactose, mannitol, glucose, microcrystalline cellulose, hydroxypropylcellulose, starch, polyvinyl pyrrolidone, aluminum magnesium silicate or the like. In the usual way, the composition may contain other additives than the inert diluent, such as a lubricant, a disintegrating agent, a stabilizing agent, a solubilizing or solubilization assisting agent or the like. If necessary, tablets or pills may be coated with a sugar or a film of a gastric or enteric substance.

~~{101}~~[110] The liquid composition for oral administration includes emulsions, solutions, suspensions, syrups and elixirs and contains a generally used inert diluent such as purified water or ethanol. In addition to the inert diluent, this composition may contain auxiliary agents such as a moistening agent, a suspending agent, a sweetener, an aromatic agent and an antiseptic agent.

~~{102}~~[111] The injections for parenteral administration includes aseptic aqueous or non-aqueous solutions, suspensions and emulsions. Examples of the diluent for use in the aqueous solutions and suspensions include distilled water for injection, physiological saline and the like. Examples of the diluent for use in the non-aqueous solutions and suspensions include propylene glycol, polyethylene glycol, plant oil (e.g., olive oil or the like), alcohol (e.g., ethanol), Polysorbate 80 and the like. Such a composition may further contain a moistening agent, an

emulsifying agent, a dispersing agent, a stabilizing agent, a solubilizing or solubilization assisting agent, an antiseptic and the like. These compositions are sterilized by filtration through a bacteria retaining filter, blending of a germicide or irradiation. Alternatively, they may be used by firstly making into sterile solid compositions and dissolving them in sterile water or a sterile solvent for injection use prior to their use.

~~{103}~~[112] The clinical dose is optionally decided by taking into consideration strength of activity of the active ingredient selected by the aforementioned screening method, symptoms, age, sex and the like of each patient to be treated.

~~{104}~~[113] For example, the dose is usually from about 0.1 to 1,000 mg, preferably from 0.1 to 100 mg, per day per adult (as 60 kg in body weight) in the case of oral administration. In the case of parenteral administration, it is from about 0.01 to 1,000 mg, preferably from 0.01 to 100 mg, per day in the form of injections.

~~Brief Description of the Drawings~~

~~{105}~~Fig. 1 is a photograph showing a result of the expression of MDPS6TSP1 in an animal cell strain using an ECL western blotting detection system, obtained in Example 6.

~~{106}~~Fig. 2 is a photograph showing a result of the detection of the activity of MDPS6TSP1 to degrade a recombinant aggrecan G1G2 using an ECL western blotting detection system, obtained in Example 7-2.

~~{107}~~Fig. 3 is a photograph showing a result of the analysis of a

~~recombinant aggrecan G1G2 degraded with MDTS6TSP1, by an anti-aggrecanase neoepitope antibody, using a western blotting detection system, obtained in Example 7-3.~~

~~{1108}Fig. 4 is an electrophoresis pattern photograph showing a result of the examination of MDTS6 mRNA expression induction by IL-1 β , obtained in Example 8.~~

~~{1109}Fig. 5 is a photograph showing a result of the detection of degradation of natural type aggrecan by MDTS6 protein, by an anti-aggrecanase neoepitope antibody, using a western blotting detection system, obtained in Example 9-2.~~

~~{1110}Fig. 6 is a graph showing a result of the detection of release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid and IL-1 β , obtained in Example 11-2.~~

~~{1111}Fig. 7 is an electrophoresis pattern photograph showing a result of the analysis of changes in gene expression of MDTS6 by RT-PCR when rabbit knee joint primary culture cells are treated with all-trans retinoic acid and IL-1 β , obtained in Example 11-3.~~

~~{1112}Fig. 8 is a graph showing that degradation and release of proteoglycan from rabbit knee joint primary culture cells by all-trans retinoic acid are inhibited by the compound A and compound B, obtained in Example 12.~~

Best Mode for Carrying Out the Invention

~~{1113}~~[114] The following describes the invention more illustratively.

~~{114}~~[115] Unless otherwise noted, experiments were carried out in accordance with gene manipulation experiment manuals such as of a known method (Sambrook, J. et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor Laboratory, NY, 1989) and the like, but the invention is not limited to the Examples.

~~{115}~~[116] (Example 1) Discovery of partial sequence of a novel ADAMTS gene MDTS6

~~{116}~~[117] A human brain cDNA library strictly fractionated by the size of insertion sequences was constructed as shown in a reference (Ohara O. et al., *DNA Res.*, 4, 53 - 59, 1997). Size distribution of cDNA fragments in these sub-libraries was from 3 kbp to 8 kbp. By deciphering 5'- and 3'-end sequences of clones constituting this library, an in-house EST data bank was constructed. A partial sequence of MDTS6 was obtained from this.

~~{117}~~[118] (Example 2) Determination of full-length ORF sequence of MDTS6

~~{118}~~[119] By determining sequences of MDTS6 cDNA clones, a sequence of from the 832nd position to the 2853rd position of SEQ ID NO:2 was obtained. The sequence of from the 1st position to the 831st position of SEQ ID NO:2 was obtained by repeating RACE (Rapid Amplification of cDNA Ends) using human brain and human placenta Marathon-Ready™ cDNA manufactured by Clontech as the template and LA-Taq™ (mfd. by Takara Shuzo) as the DNA polymerase. As a result, it was revealed that the full-length MDTS6 was a novel protein

composed of 950 amino acids as shown in SEQ ID NO:1. Its domain structure was composed of a secretion signal sequence, a pro region, a furin protease recognition sequence, a metalloprotease domain, a disintegrin domain, a thrombospondin type I repeat sequence (to be referred to as TSP-1 repeat sequence hereinafter), a domain rich in Cys residue, an intermediate region and two TSP-1 repeat sequences, in order from the N-terminus, and it was a molecule belonging to the ADAMTS family (Kuno, K. *et al.*, *J. Biol. Chem.*, 272, 556 - 562, 1997; Tang, B.L. *et al.*, *FEBS Lett.*, 445, 223 - 225, 1999).

~~{119}~~[120] (Example 3) Preparation of C-terminal FLAG addition type expression vector

~~{120}~~[121] An EBNA1 expression unit-removed expression vector pCEP4d was constructed by digesting pCEP4 (mfd. by Invitrogen) with restriction enzymes *Cla*I and *Nsi*I, blunt-ending the resulting fragments and then carrying out their autonomous ligation. This vector was digested with restriction enzymes *Nhe*I and *Bam*HI and extracted from an agarose gel to obtain a fragment of about 7.7 kbp, and a double strand of oligonucleotide obtained by annealing a nucleic acid shown by SEQ ID NO:3 and a nucleic acid shown by SEQ ID NO:4 was inserted into the fragment to select a clone having the planned sequence which was named pCEP4d-FLAG. Using this vector as the template and oligoDNA shown by SEQ ID NO:5 and oligoDNA shown by SEQ ID NO:6 as primers, PCR was carried out

using PyroBest™ DNA polymerase. The thus generated DNA fragment of about 0.4 kbp was digested with a restriction enzyme *SpeI* and inserted into pCEP4d-FLAG (about 7.7 kbp) which had been digested with *XbaI*, and a clone in which *XbaI*, *NheI*, *NotI* and *BamHI* recognition sequence cloning sites and FLAG tag were arranged in that order from the promoter as intended was selected, thereby completing pCEP4dE2-FLAG.

{121}[122] (Example 4) Construction of MDTS6 truncated protein (MDTS6TSP1) expression plasmid

{122}[123] A plasmid was constructed in the following manner for use in expressing a sequence of from the 1st position to the 583rd position of SEQ ID NO:1 (a moiety corresponding to a region containing the TSP1 repeat sequence from the N-terminus of MDTS6 (to be referred to as MDTS6TSP1 hereinafter)) as a protein in which FLAG was added to the C-terminus.

{123}[124] Firstly, a gene of from the 1st position to the 1749th position of SEQ ID NO:2 was obtained by PCR. Using oligoDNA sequences represented by SEQ ID NO:7 and SEQ ID NO:8 as primers, human placenta Marathon-Ready™ cDNA (mfd. by Clontech) as the template and LA-Taq™ (mfd. by Takara Shuzo) as DNA polymerase, a cycle of 98°C for 10 seconds and 68°C for 2 minutes was repeated 10 times after heating at 94°C for 1 minutes. Using a DNA solution prepared by 50 times-diluting this reaction solution as the template and using PyroBest™ DNA polymerase, PCR was carried out

under a condition of 94°C for 2 minutes, 40 repetitions of a cycle of 98°C for 10 seconds, 66°C for 30 seconds and 74°C for 4 minutes and subsequent 72°C for 10 minutes. The thus generated fragment of interest in which *Xba*I recognition sequence and Kozak sequence were added to the 5' side, and *Not*I recognition sequence to the 3' side, was subcloned into PCR-Blunt to confirm the sequence and then digested with restriction enzymes *Xba*I and *Not*I and inserted into the *Xba*I-*Not*I site of pCEP4dE2-FLAG to complete pCEP-MDTS6TSP1-FLAG.

{124}[125] (Example 5) Construction of MDTS6 full-length protein expression plasmid

{125}[126] A plasmid was constructed in the following manner for use in expressing a sequence of from the 1st position to the 950th position of SEQ ID NO:1 as a protein in which FLAG was added to the C-terminus.

{126}[127] Firstly, a gene of from the 1534th position to the 2850th position of SEQ ID NO:2 was obtained by PCR. Illustratively, using oligoDNA sequences represented by SEQ ID NO:9 and SEQ ID NO:10 as primers, the plasmid DNA of EST clone as the template and PyroBest™ DNA polymerase as DNA polymerase, a cycle of 98°C for 10 seconds, 50°C for 15 seconds and 72°C for 2 minutes was repeated 20 times after heating at 94°C for 1 minutes, followed by 7 minutes of reaction at 72°C. In this connection, it was able to generate

the fragment of interest by carrying out PCR using human placenta Marathon-Ready™ cDNA (mfd. by Clontech) as the template, instead of using the plasmid DNA of EST clone as the template, and using oligoDNA sequences represented by SEQ ID NO:9 and SEQ ID NO:10 as primers under a condition of 94°C for 2 minutes, 40 repetitions of a cycle of 98°C for 10 seconds and 68°C for 2 minutes and subsequent 72°C for 7 minutes. The thus generated fragment of interest in which *NotI* recognition sequence was added to the 3' side was subcloned into PCR-Blunt to confirm the sequence and used as pCRB-MDTS6-3H.

~~[127]~~[128] Making use of the presence of a *BamHI* recognition sequence in a sequence of from the 1566th position to the 1571st position of SEQ ID NO:2, pCEP-MDTS6TSP1-FLAG was digested with restriction enzymes *XbaI* and *BamHI*, and the thus generated DNA fragment of about 1.6 kbp was connected to a DNA fragment of about 1.3 kbp generated by digesting pCRB-MDTS6-3H with *BamHI* and *NotI* and inserted into the *XbaI*-*NotI* site of pCEP4dE2-FLAG to complete pCEP-MDTS6F-FLAG.

~~[128]~~[129] (Example 6) Expression of MDTS6TSP1 and MDTS6 full-length proteins by animal cell strain

~~[129]~~[130] The expression plasmid prepared in Example 4 using pCEP4dE2-FLAG as the backbone was introduced into HEK293-EBNA cell (mfd. by Invitrogen) using FuGENE™6 Transfection Reagent (mfd. by Boehringer Mannheim) in accordance with the attached instructions.

After introduction of the plasmid, the presence of the protein of interest in a culture supernatant obtained by 1 to 2 days of culturing was confirmed by western blotting using an antibody against FLAG tag added to the C-terminus (a mouse anti-FLAG monoclonal antibody (M2; mfd. by Sigma)). That is, the culture supernatant was subjected to electrophoresis using SDS/10% to 20% acrylamide gel (mfd. by Daiichi Pure Chemicals) and then transferred on a PVDF membrane using a blotting apparatus. The PVDF membrane after the transfer was subjected to blocking by adding Block Ace (mfd. by Dainippon Pharmaceutical) and then allowed to react with the mouse anti-FLAG monoclonal antibody (M2; mfd. by Sigma) and a horseradish peroxidase-labeled rabbit anti-mouse IgG polyclonal antibody (mfd. by Zymed or TAGO) in that order. Alternatively, after the blocking, it was allowed to react with biotinylated M2 antibody (mfd. by Sigma) and a streptavidine-horseradish peroxidase conjugate (mfd. by Amersham) in that order. After the reaction, expression of the protein was confirmed using an ECL western blotting detection system (mfd. by Amersham Pharmacia) (Fig. 1). Molecular weight of the expressed MDTS6TSP1 protein was smaller than the value calculated from the amino acid sequence by a factor of about 23 K. Making use of the fact that FLAG tag is added to the C-terminus of MDTS6TSP1 protein expressed by the HEK293-EBNA cell as described in the foregoing, MDTS6TSP1 protein was affinity-purified by the method of Example

7-1 and then transferred on a PVDF membrane, and the N-terminal sequence of MDTS6TSP1 protein stained with Ponceau S was determined by analyzing with Type 494 Peptide Sequencer manufactured by ABI. As a result, it was shown that it starts from the 213th position Phe of SEQ ID NO:1 and, similar to the case of other ADAMTS molecules, becomes mature protein (from 213th position to 583rd position of SEQ ID NO:1) by being cleaved at the furin protease recognition sequence existing between the pro region and metalloprotease domain. Also, the MDTS6 full-length protein was obtained in the same manner as the case of the above MDTS6TSP1 protein expression using the expression plasmid obtained in Example 5, and similar to the case of MDTS6TSP1, it was confirmed that it becomes mature protein (from 213th position to 950th position of SEQ ID NO:1) by being cleaved at the furin protease recognition sequence existing between the pro region and metalloprotease domain.

~~{130}~~[131] (Example 7) Detection of enzyme activity of MDTS6TSP1 protein expressed in animal cell host

~~{131}~~[132] (Example 7-1) Preparation of recombinant aggrecan G1G2

~~{132}~~[133] Using oligoDNA sequences represented by SEQ ID NO:11 and SEQ ID NO:12 synthesized based on the reported gene sequence of human aggrecan (Doege K. et al., *Biochem. Soc. Trans.*, 18, 200 - 202, 1990) as primers, human placenta Marathon-Ready™ cDNA as the template and PyroBest™ DNA polymerase as DNA polymerase, the

reaction of 94°C for 1 minute, 40 repetitions of a cycle of 98°C for 10 seconds and 68°C for 2 minutes, and subsequent 68°C for 7 minutes was carried out. The thus generated DNA fragment was digested with a restriction enzyme *Bam*HI and inserted into the *Bam*HI site of pCEP-SigFla, thereby completing an expression plasmid pCEP-rAgg for use in the expression of a protein in which FLAG tag is added to the N-terminus, and His tag to the C-terminus, of the globular domain 1 (G1)-globular domain 2 (G2) of human aggrecan. The pCEP-SigFla is an expression vector which is prepared by introducing double strand of the oligoDNA sequences represented by SEQ ID NO:13 and SEQ ID NO:14 into the *Hind*III-*Xho*I site of pCEP4d and has the influenza virus hemagglutinin secretion signal sequence described in the report (Guan X-M. *et al.*, *J. Biol. Chem.*, 267, 21995 - 21998, 1992), FLAG tag and *Bam*HI recognition sequence in that order downstream of the promoter.

~~[133]~~[134] The plasmid pCEP-rAgg was introduced into HEK293-EBNA cell which was subsequently cultured for 3 to 7 days, thereby effecting expression and production of the protein of interest. Purification of the protein of interest from the culture supernatant was carried out by an affinity purification making use of the addition of FLAG tag to the N-terminus. That is, the culture supernatant was applied to M2-agarose (mfd. by Sigma) packed in a column, washed with 20 mM Tris-HCl (pH 7.4)/150 mM NaCl (to be referred to as TBS hereinafter), eluted and

fractionated with 0.1 M Gly-HCl (pH 3.0) and immediately neutralized with 1 M Tris-HCl (pH 8.0).

~~{134}~~[135] (Example 7-2) Detection of recombinant aggrecan G1G2
degrading activity of MDTS6TSP1 protein

~~{135}~~[136] In Example 6, the medium 12 to 16 hours after introduction of the expression plasmid was replaced by a serum-free medium, and the culturing was continued for 32 to 36 hours to recover the culture supernatant. This culture supernatant was mixed with the recombinant aggrecan prepared in the foregoing, and the mixture was allowed to undergo the reaction at 37°C overnight, subjected to SDS-PAGE, transferred on a PVDF membrane and blocked by the method described in Example 6 and then allowed to react with an anti-Hisx6 polyclonal antibody (sc-803; mfd. by Santa Cruz Biotechnology) and a horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (mfd. by BML) in that order. After the reaction, the recombinant aggrecan was detected using an ECL western blotting system (mfd. by Amersham Pharmacia). As a result, degraded fragment of the recombinant aggrecan, which was not found in the control in which only the expression plasmid was introduced, was detected (Fig. 2).

~~{136}~~[137] (Example 7-3) Analysis by anti-aggrecanase neoepitope
antibody

~~{137}~~[138] Aggrecanase is a metalloprotease which selectively cleaves aggrecan at the site between Glu³⁷³-Ala³⁷⁴. An antibody capable of

recognizing a C-side neoepitope generated by this cleavage was prepared in accordance with a usual method by repeating immunization of mouse with a conjugate of the synthetic peptide represented by SEQ ID NO:32 and KLH, 5 times. A PVDF membrane after transfer and blocking carried out in the same manner as in Example 7-2 was allowed to react with this antibody, allowed to react with a peroxidase-labeled goat anti-mouse IgG polyclonal antibody (mfd. by Tago) and then detected using an ECL western blotting detection system (mfd. by Amersham Pharmacia). As a result, the degraded product of recombinant aggrecan generated by MDT56 reacted with the anti-aggrecanase neoepitope antibody, and molecular weight of the detected band is consistent with the molecular weight of the degraded product detected in Example 7-2 (Fig. 3). The same result was obtained by the BC-3 antibody which recognizes aggrecanase neoepitope (Hughes C.E. et al., Biochemical J., 305, 799 - 804, 1995).

{138}[139] (Example 8) Expression induction of MDT56 mRNA by IL-1

{139}[140] It is known that a mouse cell strain ATDC5 is differentiated into a chondrocyte-like cell by insulin treatment (Atsumi T. et al., Cell Differ. Dev., 30, 109 - 116, 1990). The ATDC5 cells were inoculated in 4×10^5 /well portions into an I type collagen-coated 6 well plate (mfd. by Asahi Technoglass) and cultured for 2 days using DMEM/HamF12 (1:1)/5% FCS medium, the medium was changed to DMEM/HamF12 (1:1)/5% FCS medium containing insulin (final

concentration 30 ng/ml) and 50 µg/ml of L-ascorbic acid and the culturing was continued for 5 days, and then the resulting cells were treated for 0, 1, 2, 4 or 8 hours by adding IL-1 β (final concentration 5 ng/ml). Total RNA was prepared from each of the treated groups using ISOGEN (mfd. by Nippon Gene), and RT-PCR was carried out using 1 µg portion thereof as the template and using BcaBESTTM RNA PCR Kit (mfd. by Takara Shuzo). The reverse transcription reaction was carried out using Oligo dT-Adaptor Primer as the primer in accordance with the attached instructions, and PCR was carried out using oligoDNA sequences represented by SEQ ID NO:15 and SEQ ID NO:16 as primers, which had been synthesized based on the 3' non-translation region of MDTS6, by the reaction of 94°C for 2 minutes, 40 repetitions of a cycle of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and subsequent 72°C for 7 minutes. The reaction solution was subjected to electrophoresis with 1% agarose, and densities of the thus generated bands of about 0.3 kbp were compared. As a result, it was found that expression of the MDTS6 mRNA is transiently induced by IL-1 (Fig. 4).

~~{140}~~[141] (Example 9) Degradation of natural type aggrecan by MDTS6

~~{141}~~[142] (Example 9-1) Expression of various full-length MDTS6 proteins and their recombinant aggrecan G1G2 degrading activity

~~{142}~~[143] The expression plasmid constructed using pCEP4dE2-FLAG as

the backbone was introduced into HEK293-EBNA cell (mfd. by Invitrogen) using FuGENE™6 Transfection Reagent (mfd. by Boehringer Mannheim) in accordance with the attached instructions. After introduction of the plasmid, the resulting cells were cultured overnight and washed with PBS buffer, and then the medium was changed to a serum-free medium and the culturing was continued for 2 to 3 days. The resulting culture broth was centrifuged at 9,000 rpm for 10 minutes, and the supernatant was used as the enzyme source of MDTS6. In this case, in addition to the expression plasmids described in Example 4 and Example 5, expression plasmids for three proteins, namely a protein in which the polypeptide represented by SEQ ID NO:33 was added to the C-terminus of the amino acids of from the 1st position to the 447th position of SEQ ID NO:1 (to be referred to as MDTS6Pro hereinafter), a protein in which the polypeptide represented by SEQ ID NO:33 was added to the C-terminus of the amino acids of from the 1st position to the 518th position of SEQ ID NO:1 (to be referred to as MDTS6Dis hereinafter) and a protein in which the polypeptide represented by SEQ ID NO:33 was added to the C-terminus of the amino acids of from the 1st position to the 687th position of SEQ ID NO:1 (to be referred to as MDTS6Cys hereinafter), were designed as expression plasmids for respective full-length MDTS6 proteins. That is, the MDTS6Cys expression plasmid was constructed by amplifying a gene by PCR using the

full-length protein expression plasmid constructed in Example 5 as the template and the oligoDNA sequences represented by SEQ ID NO:7 and SEQ ID NO:17 as primers and using PyroBest DNA polymerase, digesting the gene with restriction enzymes *Xba*I and *Not*I, and then inserting the resulting fragment into the *Xba*I-*Not*I site of pCEP4dE2-FLAG. Also, the MDTS6Pro expression plasmid and the MDTS6Dis expression plasmid were constructed in the same manner as the plasmid prepared using the MDTS6Cys, illustratively, by digesting respective genes amplified by PCR using PyroBest DNA polymerase with restriction enzymes *Xba*I and *Not*I, and then inserting the resulting fragments into the *Xba*I-*Not*I site of pCEP4dE2-FLAG. Provided that the oligoDNA represented by SEQ ID NO:7 and the oligoDNA represented by SEQ ID NO:34 were used in the case of the MDTS6Pro, and a combination of the oligoDNA represented by SEQ ID NO:7 and the oligoDNA represented by SEQ ID NO:35 was used in the case of the MDTS6dis, respectively.

~~[143]~~[144] Regarding protein expression of these respective MDTS6 proteins (MDTS6Cys, MDTS6Pro and MDTS6Dis), they were expressed in the same manner as the expression of MDTS6TSP1 and MDTS6 full-length proteins in an animal cell strain described in (Example 6). When the aggrecanase activity of these respective MDTS6 proteins was examined by the method of Example 7-3, the aggrecanase activity was detected in the culture supernatant in which MDTS6Cys was expressed, but the aggrecanase activity was not detected in

the culture supernatants in which MDTS6Pro and MDTS6Dis were expressed. In this connection, molecular weight of the expressed main protein was smaller than the value calculated from the amino acid sequence by a factor of about 23 K and, similar to the case of MDTS6TSP1 described in Example 6, it was mature protein in which the pro region was cleaved and removed at the furin protease recognition sequence. As a result, it was revealed that the first TSP-1 repeat sequence counting from the N-terminus is essential for exerting the aggrecanase activity of MDTS6.

~~{144}~~[145] (Example 9-2) Degradation of natural type aggrecan

~~{145}~~[146] A 90 μ l portion of the MDTS6 enzyme solution prepared in Example 9-1 was mixed with a solution of 10 μ g natural type aggrecan (mfd. by Seikagaku Kogyo)/10 μ l TBS in a test tube and allowed to undergo the reaction at 37°C overnight. This reaction product was dried up using SpeedVac and then dissolved in 100 μ l of 10 mM Tris-acetate buffer (pH 7.6) containing 0.06 unit of Chondroitinase ABC (mfd. by Seikagaku Kogyo), 0.024 unit of keratanase I (mfd. by Seikagaku Kogyo), 0.0004 unit of keratanase II (mfd. by Seikagaku Kogyo), 5 μ M of PMSF and 10 mM of EDTA, and the solution was allowed to undergo the reaction at 37°C overnight. A portion of this reaction solution was subjected to SDS-PAGE and then the product was detected using the mouse anti-aggrecanase neoepitope antibody as shown in Example 7-3. In this case, the

peroxidase-labeled goat anti-mouse IgG polyclonal antibody used was a preparation manufactured by Biosource. The same result was obtained by the BC-3 antibody which recognizes aggrecanase neoepitope (Hughes C.E. et al., *Biochemical J.*, 305, 799 - 804, 1995).

~~[146]~~[147] As a result, a band of 80 to 90 KDa was detected in the case of MDTS6Cys in addition to a band of about 150 KDa. This degradation pattern is consistent with the pattern of main molecules (all generated by aggrecanase degradation) found in the joint synovial fluids of patients of joint diseases including OA and RA (Sandy J.D. et al., *J. Clin. Invest.*, 89, 1512 - 1516, 1992; Lohmander L.S. et al., *Arthritis Rheum.*, 36, 1214 - 1222, 1993) and also is consistent with the pattern of main molecules having aggrecanase neoepitope which are generated after 12 to 24 hours of treatment with IL-1 and retinoic acid in an explant culture system of human knee joint cartilage (Little C.B. et al., *Biochemical J.*, 344, 61 - 68, 1999) (Fig. 5).

~~[147]~~[148] (Example 10) Screening system of substances which modify the aggrecanase activity

~~[148]~~[149] (Example 10-1) Preparation of MDTS6Cys and substrate

~~[149]~~[150] It was confirmed using the western blotting method shown in Example 9-2 that the recombinant aggrecan G1G2 and the natural type aggrecan are cleaved off at the site between Glu³⁷³-Ala³⁷⁴ (to be referred to as "aggrecanase site" hereinafter") by MDTS6Cys

without purification but as the culture supernatant prepared by the method of Example 9-1. Also, the cleavage at the "aggrecanase site" was observed when the culturing in Example 9-1 was continued with the 10% FBS-containing medium without changing to the serum-free medium. Accordingly, the recombinant aggrecan G1G2 prepared in Example 7-1 was used as the substrate.

{150}[151] (Example 10-2) Screening system

{151}[152] Though the screening can be carried out by the western blotting-aided method shown in Example 7-2 using the recombinant aggrecan or natural type aggrecan as the substrate, the following ELISA system was constructed for screening more larger number of compounds to be tested.

{152}[153] An MDTs6Cys culture supernatant, the recombinant aggrecan G1G2 and a compound to be tested were mixed and allowed to undergo the reaction at 37°C for several hours, the resulting product was adhered to a 96 well plate (Nunc-Immuno™ Plate MaxiSorp™ Surface # 439454; mfd. by Nunc), blocked with 1% BSA/TBS solution and then allowed to react with a mouse anti-aggrecanase neoepitope antibody and an HRP-anti-mouse IgG antibody conjugate (mfd. by Biosource) in that order, and then the detection was carried out using TMB Peroxidase EIA Substrate Kit (mfd. by Bio-Rad) under the conditions described in the attached instructions to calculate the aggrecanase activity inhibiting strength of the compound to be tested using the coloring inhibition as a marker. Also, as a

modified method thereof, the recombinant aggrecan was adhered to the 96 well plate (mfd. by Nunc) and blocked with 1% BSA/TBS solution in advance and then an MDTs6Cys culture supernatant and a compound to be tested were added thereto and allowed to undergo the reaction at 37°C for several hours, the resulting product was allowed to react with a mouse anti-aggrecanase neoepitope antibody and an HRP-anti-mouse IgG antibody conjugate (mfd. by Biosource) in that order, and then the detection was carried out using TMB Peroxidase EIA Substrate Kit (mfd. by Bio-Rad) to calculate the aggrecanase activity inhibiting strength of the compound to be tested using the coloring inhibition as a marker. The criterion to screen a substance which inhibits the aggrecanase activity is preferably 10 μM or less, more preferably 1.0 μM or less, as inhibition activity strength (IC_{50}).

~~[153]~~[154] By this screening system, it was able to select the aforementioned compound A, compound B, compound C and compound D. The aggrecanase activity inhibition strength (IC_{50}) was 0.6 μM for the compound A, 1.0 μM for the compound B, 2.9 μM for the compound C and 2.7 μM for the compound D.

~~[154]~~[155] In this connection, the compound A, compound B, compound C and compound D were synthesized in the same manner as the production method described in PCT publication number WO 90/05719. The mass spectrum of respective compounds is as follows. The compound A is $\text{MS} = 426 (\text{MH}^+)$, the compound B is $\text{MS} = 396 (\text{MH}^+)$, the

compound C is MS = 502 (MH⁺) and the compound D is MS = 440 (MH⁺).

~~{155}~~[156] (Example 11)

~~{156}~~[157] (Example 11-1) Preparation of rabbit knee joint cartilage
primary culture cells

~~{157}~~[158] After killing a rabbit (Japanese white species, male, 1.0 to 1.5 kg) under excess anesthesia, a knee joint was excised and the cartilage layer on the joint surface was removed and finely cut using a surgical knife. The cut pieces were treated with trypsin-EDTA (0.25%-1 mM; mfd. by GIBCO-BRL) at 37°C for 1 hour and then centrifuged at 1,500 rpm for 5 minutes, and the resulting precipitate was washed with DMEM. This was treated with collagenase A (0.15%; Boehringer-Mannheim)/DMEM at 37°C for 3 to 4 hours, and then a nylon mesh filter (100 μm, mfd. by Falcon)-passed fraction was centrifuged at 1,500 rpm for 5 minutes to effect precipitation of cartilage cells. The cells were thoroughly washed with DMEM/10% FBS medium, suspended in DMEM/10% FBS medium to a density of 2×10^5 cells/ml and then inoculated in 200 μl/well portions into an I type collagen-coated 96 well plate (mfd. by Asahi Technoglass). Three days thereafter, the medium was changed to 200 μl of DMEM/10% FBS medium containing 50 μg/ml of ascorbic acid (ascorbic acid medium hereinafter), and the culturing was continued for 3 days. When an I type collagen-coated 6 well plate (Asahi Technoglass) was used, the cell suspension was inoculated in 6 ml/well portions and cultured by

carrying out the same medium exchange. These cells were used in the following test.

~~{158}~~[159] (Example 11-2) Proteoglycan degradation of rabbit knee joint cartilage primary culture cells

~~{159}~~[160] The rabbit knee joint cartilage primary culture cells of 96 well plate described in Example 11-1 were cultured for 2 days using 200 μ l of the ascorbic acid medium supplemented with 10 μ Ci/ml in final concentration of $\text{Na}_2^{35}\text{SO}_4$ and labeled therewith, washed 3 times with 200 μ l of the ascorbic acid medium and then cultured for 1 day using 200 μ l of the ascorbic acid medium. After stimulation with IL-1 β or all-trans retinoic acid and subsequent 0, 24, and 48 hours of culturing, the culture supernatants were recovered in 20 μ l portions, and the radioactivity was measured using Top Count (mfd. by Packard). As a result, increase in the radioactivity, namely release of proteoglycan, was observed by 0.01 to 10 ng/ml of IL-1 β stimulation, and increase in the concentration-dependent and strong radioactivity, namely release of proteoglycan, was observed by 0.1 to 10 μ M of all-trans retinoic acid stimulation (Fig. 6).

~~{160}~~[161] (Example 11-3) Induction of MDTS6 mRNA expression

~~{161}~~[162] After 3 days of culturing of the rabbit knee joint cartilage primary culture cells of 6 well plate described in Example 11-1 by changing the medium to ascorbic acid medium, 10 ng/ml of IL-1 β or

10 μ M of all-trans retinoic acid was added thereto, and total RNA samples 2 and 6 hours thereafter were prepared using ISOGEN (mfd. by Nippon Gene) in accordance with the attached instructions. Each of the samples was treated with DNase I (mfd. by Nippon Gene), subjected to phenol/chloroform treatment and then recovered by ethanol precipitation, and the thus purified total RNA was dissolved in DEPC-treated sterile water. Using random hexamers as primers, 1 μ g of this total RNA was subjected to reverse transcription reaction and RNase H treatment using Thermoscript™ RT-PCR System (mfd. by GIBCO-BRL, catalog number 11146-016) in accordance with the attached instructions, and the product was diluted 10 times with sterile water and used as a cDNA sample. Using 5 μ l of each of the thus obtained cDNA samples as the template and the oligoDNA sequences represented by SEQ ID NO:18 and SEQ ID NO:19 as primers, PCR was carried out under a condition of 94°C for 2 minutes, 45 repetitions of a cycle of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds, and subsequent 72°C for 10 minutes. The reaction products were subjected to 2% agarose electrophoresis, and densities of the generated DNA fragments were compared. As a result, expression of the MDTS6 mRNA was induced by IL-1 β and all-trans retinoic acid, and the expression strength correlated with the degree of proteoglycan degradation described in Example 11-2 (Fig. 7).

~~{162}~~{163} (Example 12) Inhibition of proteoglycan degradation in rabbit knee joint cartilage primary culture cells by substances which inhibit the aggrecanase activity

~~{163}~~{164} Each of the compounds A, B, C and D selected by the screening system of Example 10-2 was added to the proteoglycan degradation system of rabbit knee joint cartilage primary culture cells just before the stimulation with 10 μM of all-trans retinoic acid, and their proteoglycan degradation inhibitory activities were examined. As a result, the compounds A and B showed the inhibition of proteoglycan degradation in a concentration-dependent manner (Fig. 8). The proteoglycan degradation inhibition action (IC_{50}) of the compounds C and D was 6.3 μM for the compound C and 4.1 μM for the compound D. On the other hand, the proteoglycan degradation inhibition action was not observed by compounds which have the same hydroxamic acid backbone but show a weak aggrecanase activity inhibition, even at a concentration of 100 μM .

~~{164}~~{165} (Example 13) Analysis of MDTS6 promoter region DNA sequence

~~{165}~~{166} A DNA fragment corresponding to the promoter region of MDTS6 was amplified using PCR from GenomeWalker DNA Sca I Libraries (Genome Walker™ Kits, CLONTECH catalog number K1803-1). OligoDNA sequences of the adapter primers AP-1 (SEQ ID NO:20) and AP-2 (SEQ ID NO:21) attached to the kit were used as forward primers, and the oligoDNA sequences of SEQ ID NO:22 and SEQ ID NO:23 as reverse

primers. The illustrative method was as described in the instructions attached to the kit, but TAKARA LA Taq (TAKARA LA TaqTM, catalog number RR002A) was used in the PCR. The first PCR was carried out using the oligoDNA sequences of SEQ ID NO:20 and SEQ ID NO:22 as primers under a condition of 7 repetitions of a cycle of 98°C for 5 seconds and 72°C for 3 minutes, 32 repetitions of a cycle of 98°C for 5 seconds and 67°C for 3 minutes, and 67°C for 4 minutes. The second PCR was carried out under the same conditions using 5 µl of a solution prepared by diluting the reaction solution of the first reaction 50 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) as the template, and the oligoDNA sequences of SEQ ID NO:21 and SEQ ID NO:23 as primers. When the thus amplified DNA fragment of about 3.7 kbp was directly subjected to sequence analysis by dideoxy terminator method using ABI377 DNA Sequencer (Applied Biosystems Inc.), DNA sequences of about 2.2 kbp, 0.36 kbp and 0.8 kbp divided by two un-decipherable gaps were found.

[166][167] Next, in order to decipher sequences of these two gap moieties which were unable to decipher by the direct analysis of the PCR-amplified DNA fragment, this DNA fragment was subcloned and the DNA nucleotide sequence was determined. As a result, sequences of the gap moieties were different in the determined 8 clones (SEQ ID NOs:24, 25, 26, 27, 28, 29, 30 and 31), so that the presence of gene polymorphism was suggested. In this connection,

pZErOTM-2 vector (Zero Background/Kan Cloning Kit, mfd. by Invitrogen, catalog number K2600-01) was used as the cloning vector, and the subcloning was carried out in accordance with the attached instructions.

~~{167}~~[168] A plasmid prepared by inserting the above DNA fragment into the *KpnI*-*XhoI* site of a reporter plasmid pGV-B2 (mfd. by Toyo Ink) was introduced into HEK293 cell using FuGene-6, and the luciferase activity after 28 or 48 hours of culturing under usual culturing conditions was measured using PicaGene coloring kit (mfd. by Toyo Ink, catalog number PGK-L100). In this case, the measured value was normalized by the activity value of β -gal expressed by a simultaneously introduced β -gal expression plasmid pCH110 (Amersham Pharmacia Biotech, catalog number 27-4508-01). The β -gal activity was measured using Galacto-Light Plus Kit (mfd. by TROPIX, catalog number BL300P). As a result, distinct increase in the luciferase activity which cannot be found in the original plasmid pGV-B2 was observed. This result indicates that the promoter activity is present in the above DNA fragment.

~~{168}~~[169] (Example 14) Expression of MDTS6 in joint tissue of osteoarthritis patient

~~{169}~~[170] Total RNA was prepared from an affected part of a knee joint cartilage of an osteoarthritis patient (Adams M.E. *et al.*, *Anal. Biochem.*, 202, 89 - 95, 1992), and the presence of MDTS6 mRNA was confirmed by carrying out RT-PCR using this as the template in

accordance with Example 11-3. Also, the presence of MDTS6 protein in synovial membrane and macrophage was confirmed by carrying out immunological tissue staining using a mouse anti-human MDTS6-specific polyclonal antibody.

~~{170}~~[171] In this connection, the mouse anti-human MDTS6-specific polyclonal antibody was prepared in the following manner. Firstly, the MDTS6TSP1 protein prepared in Example 6 was conjugated with KLH, and mice were immunized with this 4 to 5 times to obtain an antiserum sample. Next, IgG was prepared from this antiserum using Protein G Sepharose 4 Fast Flow (mfd. by Amersham Pharmacia Biotech) in accordance with the attached instructions. Next, a column in which the human MDTS6TSP1 protein was fixed to CNBr-activated Sepharose 4 Fast Flow (mfd. by Amersham Pharmacia Biotech) was prepared in accordance with the attached instructions. Thereafter, a fraction was prepared which binds to this column but does not bind to a column immobilized with human ADAMTS4TSP1 protein (aggrecanase-1; Tortorella M.D. et al., *Science*, 284, 1664 - 1666, 1999), METH-1TSP1 protein (Vazquez F. et al., *J. Biol. Chem.*, 274, 23349 - 57, 1999) or METH-2TSP1 protein (Vazquez F. et al., *J. Biol. Chem.*, 274, 23349 - 57, 1999).

Industrial Applicability

~~{171}~~[172] The "joint disease aggrecanase" obtained by the invention is characterized in that it can be used for the screening of a substance which significantly inhibits the aggrecanase (a compound,

a peptide, an antibody or an antibody fragment), because it has an aggrecanase activity. Regarding the medicinal use of the substance which significantly inhibits the "joint disease aggrecanase", it is suggested that it is effective in preventing and treating diseases which are caused by abnormalities (e.g., acceleration, reduction, degeneration and the like) of the aggrecanase activity or in which the abnormalities are expressed to cause complications, particularly joint diseases as diseases which show acceleration of proteoglycan degradation, most particularly osteoarthritis.

{172}[173] Also, the promoter gene of the "joint disease aggrecanase" of the invention is characterized in that it can be used for the screening of a substance which inhibits promoter activity of the gene (a compound, a peptide, an antibody or an antibody fragment). As the use of the substance which inhibits the promoter activity, it is suggested that it is effective in preventing and treating diseases caused by inhibition of the promoter activity, particularly joint diseases as diseases which show acceleration of proteoglycan degradation, most particularly osteoarthritis. In addition, since two or more mutants are present in the promoter gene, they can be used for their correlation analysis with these diseases.